EFFECTS OF SUBLETHAL COPPER EXPOSURE ON ESCAPE BEHAVIOR AND GROWTH OF *Rana pipiens* TADPOLES

Melinda Redick, B.S.

Thesis Prepared for the Degree of

MASTER OF SCIENCE

UNIVERSITY OF NORTH TEXAS

May 2002

APPROVED:

Thomas W. LaPoint, Major Professor and Director
of the Institute of Applied Science
William T. Waller, Committee Member
James Kennedy, Committee Member
C. Neal Tate, Dean of the Robert B. Toulouse
School of Graduate Studies

Redick, Melinda, Effects of Sublethal Copper Exposure on Escape Behavior and Growth of *Rana pipiens* Tadpoles. Master of Science (Environmental Science), May 2002, 57 pp., 5 tables, 12 illustrations, bibliography, 74 titles.

This research is designed to test how sublethal exposure to copper affects tadpole predator-escape behavior and how quickly tadpoles recover. After exposure, tadpoles were separated. Escape behavior was recorded for two-thirds of exposed tadpoles while one-third of the exposed population was measured weekly to determine growth and recovery.

Control tadpoles were consumed within 15 minutes whereas those exposed to higher concentrations were consumed at a slower rate, which does not support the hypotheses. Although the rate of predation was lower, tadpoles exposed to higher Cu concentrations were on average, 1.47 cm in total body length. Those exposed to 0.93 mg/L averaged 0.86 cm. After being placed into clean water, treatment tadpoles recovered after 20 days.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF ILLUSTRATIONS

Figure Page

CHAPTER 1

INTRODUCTION

Amphibian As Environmental Indicators

Class Amphibia is comprised of three major orders: frogs and toads (Anura), s alamanders (Caudata), and caecilians (Gymnophiona), although approximately 90% are anurans. Amphibians are found in nearly every environment from deserts to tropics and even on mountaintops. Considered keystone members of ecosystems, amphibians often constitute the largest sum of vertebrate biomass in an ecosystem due to sheer densities. For example, in a shallow 750 m^2 rain-filled pool, an estimated 45,000 Mexican tree frogs (*Smilisca baudinii*) gathered to breed (Gadow, 1908). *Eurycea nana*, a small salamander inhabiting algal mats in Texas, has been observed at densities of 116/m^2 (Tupa, 1976). Roles amphibians play in an ecosystem range from top carnivores-consuming invertebrates, to the main source of food for birds, mammals and aquatic insects (Blaustine, 1990). High densities of animals that act as both predator and prey suggest amphibians are vital links in the food chain. Massive amounts of algae-consuming tadpoles are found in ponds every spring creating a link by which energy is transferred from aquatic to terrestrial environments by way of predation. Adult amphibians are typically carnivorous, functioning to control invertebrate populations. Due to the vital role amphibians play in an ecosystem, a decline or loss may conceivably cause serious ramifications throughout entire ecosystems.

Not only are amphibians an integral part of every ecosystem they inhabit, they also function as valuable biological indicators of environmental stresses due to the varied habitats they occupy throughout their life cycle and the lack of defense

mechanisms necessary to protect them from environmental changes. From embryo to adult, amphibians occupy two opposing environments and display contrary eating habits. Typical amphibian existence begins in an aquatic environment. The eggs are aquatic, covered in a semi-permeable jelly coating, but are devoid of a protective shell. Hatched tadpoles are completely aquatic and consume only plant material. Skin of larval amphibians is highly permeable and has no protective scaling to shield organisms from chemical exposure. Once fully metamorphosed, amphibians become completely carnivorous and mostly terrestrial but adults typically don't stray more than 2 km from where they hatched. The many ecosystems that amphibians occupy, the fact that they have extremely permeable skin, and relatively small home ranges makes them ideal monitors for local conditions (Lefcort 1998).

Anuran Decline

Anuran populations have declined and disappeared throughout much of the world (Halliday, 1998). A catalyst to indepth research on amphibian populations was the decline and later disappearance of the golden toad (*Bufo periglenes*) native to Monteverde Costa Rica and the gastric brooding frog (*Rheobatrachus sulus*) native to rainforests in southeastern Australia (Phillips, 1990). Since the early 1980s, when amphibians came under increasing scientific investigation, several species have become extinct locally and some globally. Scientists studying anurans in the Oregon Cascade Range have documented disappearances or declines of cascade frogs (*Rana cascadae*), red-legged frogs (*Rana aurora*) and spotted frogs (*Rana pretiosa*). California is also experiencing declines in mountain yellow-legged frog (*Rana mucosa*), Yosemite toad

(*Bufo canorus*), foothill yellow-legged frog (*Rana boylei*) and the California red-legged frog (*Rana aurora*)(Blaustine, 1995).

The decrease in amphibian numbers has prompted investigations of biotic and abiotic factors that may act as the primary culprit or as part of a synergistic effect of many factors. Proposed hypotheses formulated to explain decreasing numbers include: habitat destruction, climatic change, predator introductions, parasitic interactions, UV radiation, heavy metal exposure, and herbicide/pesticide use. As population decline has been observed globally, including pristine environments that have yet to experience direct anthropocentric activities, no singular factor would appear to explain species declines. According to fossil record, amphibians have acclimated to global changes and predation pressures for over 300 million years, which suggests decline is due to anthropocentric activity changes (Murphy, 2000).

Previous studies

Currently, toxicological information involving amphibians is limited. A review of Wildlife Review and Sports Fisheries abstracts ranging from 1972 to 1998 revealed 12,727 titles involving vertebrates and toxicology. Only 3.5% represented amphibians or reptiles (McDiarmid, 2000). Of the 4500 amphibian species, 6 are commonly represented in toxicity tests. *Xenopus laevis* is the organism of choice in over half the tests most likely due to the frog embryo teratogenesis assay *Xenopus* (FETAX) method being the only standard method of testing on amphibians. Like most model organisms, *Xenopus* was chosen for its ease of culturing in the laboratory and rapid embryological development. Although this method is useful in determining the ability of a chemical to instigate teratagenic effects, it was never intended to represent amphibians. Any

reliance on these results as representative of frogs is questionable. Model organisms should be representative of a larger group. *Xenopus* is a highly specialized, completely aquatic frog that only represents a small percentage of frogs.

Through experimentation, scientists have found different stages of amphibian development are susceptible to various stressors. Eggs are particularly sensitive to ultraviolet B radiation (Langhelle, 1999). Particularly affected are amphibians located in higher altitudes of mountainous regions (Fite, 1998). Those in tadpole stages are prone to predation and water pollution stemming from agricultural and industrial activities. Adult amphibians are influenced to a greater extent by air pollution (Blaustein, 1995) and human predation (Phillips, 1990). Scientists have several theories as to why amphibians are declining, but there is no universal answer. Amphibians in regions of high altitude are susceptible to higher amount of ultraviolet radiation, which can cause irreparable changes to DNA (Langhelle, 1999). The bacterium *Aeromonas hydrophila* has been known to wreak havoc on entire amphibian populations in Colorado (Blaustein 1995). Cascade frogs and western toads are particularly susceptible to infection by *Saprolegnia,* a fungus that kills whole clutches of eggs as well as tadpoles (Blaustein, 1995). Trematodes cause hind leg malformations in developing tadpoles, which affects mobility (Pieter, 1999). Although several mechanisms appear to play a role in the global amphibian decline, it is unknown whether or not they act alone.

Sublethal effects of exposure may have significant consequence on population but has been poorly studied (McDiarmid, 2000). Frequently, negative influences leave the affected animals less fit for survival. Amphibians affected by ultraviolet radiation as an embryo, may be more prone to lethal bacterial infections during later life stages

(Blaustein, 1995). Common tadpole responses to sublethal environmental pollution especially heavy metal and pesticide exposure, include tail kinking and erratic swimming, which is suspected to cause an increased larval predation rate (Lefcort, 1998). Amphibians with longer larval periods may be particularly susceptible to predation as predator contact time increases.

Predatory fish (Holomuzke, 1995), insects from the orders Hemiptera (Kehr, 1991), and Odonata (Caldwell 1980), crayfish (Axelsson, 1995) and aquatic spiders (Bleckmann, 1987) consume amphibian larvae as a regular part of their diet. Many predators including bluegill sunfish (*Lepomis macrochirus)* detect prey by movement. Some movement, even sinking, must occur for *L. macrochirus* to view an organism as prey (O'brien, 1976). A significant anti-predatory tadpole response is to cease movement decreasing the likelihood of detection by the predator. It is reasonable to conclude if exposure to environmental pollutants cause changes in movement, changes in predation rates will result.

CHAPTER 2

LITERATURE REVIEW

Anuran Development

In general, amphibians exhibit high fecundity and low parental care and ovipositioning marks the end of parental involvement. Depending on temperature, the embryonic phase lasts 3-6 days after oviposition (Duellman, 1994). Hatching enzymes, secreted from glands located over the snout and nape, are capable of dissolving the gelatinous egg (Duellman, 1994). The tadpole then wiggles free of the egg mass and enters the free-swimming larval phase.

Aside from the genus *Leptodactylidae*, anuran larvae are completely aquatic. Movement is a rhythmic motion of the tail coordinated by an interneuronal network that coordinates synchronizing motor neurons (Burggren, 1992). Like fishes, anuran larvae have lateral lines, which assist in navigation and balance maintenance. Lateral lines are composed of mechanoreceptors and chemoreceptors along the surface of the head and body and facilitate navigation in murky waters and detection of pressure changes in surrounding waters, which may indicate predator presence (Duellman, 1994).

Anuran larvae respire through transdermal respiration and the use of a buccal pump mechanism. Water taken in through the mouth and nostrils is pumped through the pharyngeal cavity, over gills, into the operculum chamber, then out through spiracles (Henry, 2000). Dissolved oxygen concentrations determine gill size. In oxygen rich waters, anuran larvae respiration is primarily transdermal so gill surface area is relatively small. Anurans residing in habitats with lower oxygen content generally develop gills with more branches, hence more surface area for gas exchange (Branch, 1977). A

common response to oxygen stress is gulping air from the surface, although only amphibians with developed lungs benefit from this behavior. *Bufo* tadpoles don't have lungs and must swim at the surface to absorb oxygen cutaneously (Duellman, 1994).

Respiration across gill surfaces is primarily a function of diffusion rate. Tadpoles to some extent regulate transdermal transfer by changing dermal folding to increase or decrease surface area or by regulating mucous secretion viscosity (Henry, 2000). Gills also play an active role in ion exchange, which may predispose them to certain chemical exposure.

Transformation from larval to juvenile stages is triggered by thyroxine, synthesized by the thyroid gland (Gundernatsch, 1912). During metamorphosis extreme systematical changes involving the respiratory and digestive systems, as well as physical changes involving the pelvic girdle, jaw, and cranium occur during a relatively short amount of time (Henry, 2000). Amphibians do not eat during metamorphosis. The tail is resorbed over several days providing nutrition. Due to many changes occurring in a relatively short time span, anurans are particularly vulnerable to chemical exposure as well as predation by aquatic and terrestrial predators. Complete tail absorption ends the larval stage and marks the beginning of the juvenile stage.

Unfavorable conditions may prematurely trigger hormonal and biochemical changes necessary for metamorphosis, which would remove the tadpole from the stressful environment. For this reason, size is a poor indicator of larval development. Traditionally larger anurans are thought to be older. Skeletochronological studies of toads (*B. americanus*) indicate this is not the case (Acker, 1986). When tadpoles

metamorph prematurely, they usually require more time to become sexually mature than tadpoles remaining in the larval stage for longer periods of time.

The Northern Leopard Frog *(Rana pipiens* Shreber*)* follows typical anuran life history patterns. Embryos and larval stages of *R.. pipiens* are completely aquatic whereas adult stages are mostly terrestrial.

Rana pipiens

The usual breeding season for *Rana pipiens* lasts 10- 30 days each spring season (Gilbert 1994). Depending on the altitude and temperatures, breeding season may start as early as March or as late as May (Fichter, 1964). Mature males gather and call for mates at the beginning of each spawning season. Mature females are drawn to the call, amplexus occurs and clutches of eggs are deposited in shallow water usually less than 40 cm below the surface (Merril, 1977). Each clutch is deposited in one large mass and may contain from 1 to 80,000 eggs (Jorgensen, 1992) but less than 5% of eggs will survive to metamorphosis (Henry, 2000).

Growth of *R. pipiens* in the larval stage is dependent upon environmental factors such as temperature, food availability and community structure, although R. *pipiens* size within a population of identical age are known to be highly variable. This has been attributed to food availability but also a growth inhibiting substance secreted by larger tadpoles and tadpoles under density stress. It was traditionally thought that larger tadpoles out-compete smaller ones, restricting food intake. Experiments examining growth rates of *R. pipiens* tadpoles in water where crowding has previously occurred,

demonstrate reduced growth rates indicating residual growth inhibitors present in the environment (Richards, 1958).

Size rather than chronological time determine sexual maturity and fecundity of *R. pipiens.* A length of 60 mm must be reached before *R. pipiens* reach sexual maturity (Gilbert, 1994). In the Richelieu River, this length is reached by two years of age (Gilbert, 1994) but in areas of varying food availability and temperature, sexual maturity may be reached from 1-3 years of age (Baxter, 1952). Size is also positively correlated with the number of eggs found in each clutch. A mature female longer than 90 mm typically produces over a thousand more eggs than a mature female less than 70 mm in length (Gilhen, 1984).

A negative correlation appears to exist between tadpole size and heavy metal exposure. A hypothesis that aluminum contamination affects predation detection, escape behavior and tadpole size was supported by Jung and Jagoe (1995). If side effects of copper exposure are similar, it is reasonable to conclude at the very least, clutch size will decrease affecting population numbers.

Copper In The Environment

Copper is a moderately soluble trace element, which plays a vital role in metabolic function for both prokaryotic and eucaryotic cells (Forstner, 1979). Naturally occurring, copper is found in the geologic formations of the Earth's crust. Thus bioavailibility depends on environmental factors such as climate, pH, soil and sediment composition, water hardness and organic content (Flemming, 1989)

Before the industrial revolution, copper found in the environment occurred naturally, caused by weathering of parent rock containing the element. Currently, Cu levels are increasing, exceeding expected background levels due predominantly to anthropogenic activities. The annual world production of Cu has increased from $5.8X10^9$ Kg in 1968 (Forstner, 1979) to $8.3 X 10^9$ Kg in 1985 (Bowen, 1985). Runoff from mine tailings alone contributes an estimated 314 million tons to aquatic habitat annually (Gottschalk, 1995). Other activities introducing Cu to soil and sediments include smelting as well as industrial and domestic waste emission (Forstner, 1979), and the application of fertilizer and sewage sludge (ASTDR, 1990).

Although sediments act as a sink for heavy metals, Cu is considered moderately soluble in water. Concentrations up to 18 ppm have been detected in drinking water (ASTM, 1994). Anthropogenic contributions of Cu to aquatic environments include the direct application of algicides (MacKenthun, 1952) and molluscicides (Cheng, 1997) to surface water. One of the primary sources of copper to aquatic habitat is through effluent of domestic wastewater treatment facilities to receiving systems (Nriagu, 1988). Treatment of influent removes only 55 – 90% dissolved copper before then being discharged back into the environment (Pickering et al. 1979).

Copper Toxicity

When copper is emitted to an aquatic system, there is an immediate increase in total metals. However, total metal concentrations are not indicative of toxicological response as several environmental factors render metals unavailable for biological reaction. Fulvic and humic acids chelate metal molecules upon contact removing them

from solution. Sediment particles bind free metal ions to cation exchange sites, removing metals from solution (Lanno, 1999). This process is reversible, however meaning under certain conditions sediments may act as a source as well as a sink for metal contamination.

Environmentally available free metal ions are generally absorbed through digestive or respiratory systems of aquatic organisms. Metals such as copper and zinc are micronutrients necessary for proper cellular function. Although excessive copper may, under certain circumstances become toxic, copper deficiencies carry equally severe consequences. In the presence of copper, organisms produce metallothionein (MT), a sulfhydryl-rich, low molecular weight, metal binding protein (Sanders, 1996). MT not only acts to detoxify copper at the cellular level, it also acts as a storage facility for essential metals (Lanno 1999). Only when copper exposure exceeds the binding potential of MT will toxic effects result.

Acute toxicity caused by excessive copper exposure affects an organisms ability to properly osmoregulate by enlarging leak pathways by which salts are lost to the environment rather than accumulated and utilized by the organism (Linder, 2000). The nervous system is controlled by ion flow across cell membranes through Na and K channels. When these salts are lost to the environment, nerve transmission is hindered thereby impairing the entire nervous system.

Impairment of ionic flow across cell membranes alters normal neural, as well as muscular function. Kaplan and Yoh (1961) performed laboratory experiments using adult *R. pipiens* exposed to copper sulfate solution. One noticeable effect was a decrease

in neuromuscular coordination and reaction time after a 48-hour exposure. *R. pipiens* surviving after 72 hours were flaccid and unresponsive to external stimuli.

Morphological Response

Exposure to environmental contaminants in developmental stages causes morphological and teratogenic deformities in amphibians. Toxicology experiments have been performed on embryo, larval and adult amphibian phases with the general consensus being larval amphibians are most sensitive to contaminants. Eggs are contained within a vitelline jelly membrane that is suspected to protect them from toxins (Lande, 1973). When the jelly coating is removed prior to experimentation, embryos are equally as sensitive as tadpoles to copper solutions (Birge, 1979)

Larval amphibians are more susceptible to deformities than adult amphibians due to the twenty-nine distinct morphological and anatomical changes (Gosner, 1960) they undergo before entering adult life stages. One of the most common tadpole deformities is a tail kink. Cooke (1981) found heavy metal exposure to larval amphibians increased the incidence of tail kinks in a laboratory population. Gottschalk's (1995) study exposing tadpoles to various concentrations of copper and zinc supports Cooke's original finding. Rowe (1996) found deformities of the mouth and teeth in amphibians residing in metal contaminated ponds. Although these deformities won't cause mortality alone, they do equate to elevated maintenance costs, which may lead to decreased amphibian populations.

Behavioral Response

Altered behaviors stemming from contaminant exposure may cause increased predation. It has been hypothesized that metals interfere with chemodetection of predators, which may alter predator avoidance behaviors. One of the most common anti-predatory behavioral responses is fleeing to refuge and reducing activity levels. Lefcort (1998) found after exposure to Pb or Zn *Rana luteiventris* tadpoles failed to take evasive action. *Hyla cinerea* tadpoles exposed to Al solutions were more susceptible to predation by larval dragonflies (Jung, 1995). Although metal contaminants are not directly responsible for tadpole mortality, they may induce changes in behavior or physical abilities that lead to population decline.

Lepomis macrochirus

Bluegill sunfish (*Lepomis macrochirus* Rafinesque) are one of the most common centrarchids distributed throughout the United States. They are considered planktivorous fish but are known to consume vegetation as well as aquatic insects, tadpoles and smaller fish. Aquaculturists often rear bluegill as feeder fish for larger piscivorous fish such as bass due to the ease of population maintenance and successful adaptation to new environments (Bryan, 1994).

In natural ecosystems where *L. macrochirus* are affected by both predator and prey relationships, smaller *L macrochirus* are typically found in the littoral zone amongst the vegetation (Werner, 1988). Although predation rate of *L. machrochirus* decreases as vegetation complexity increases, the littoral zones are not as conducive to prey capture as lotic zones. While a larger number and variety of prey items exist at higher macrophyte

levels, the types of prey organisms present are fairly cryptic in appearance and move slowly if at all, failing to attract the attention of *L. macrochirus* (Crowder, 1982).

Previous studies indicate *L. macrochirus* are attracted to prey based on behavior rather than size. It is clear that some motion is necessary for *L. macrochirus* to view an organism as prey, although increased motion has no effect on the rate of predation (O'Brien, 1976). When given an option between large nonmoving prey and small active prey, *L. macrochirus* consume the smaller prey significantly more than larger prey. Based on optimal foraging models for centrarchids, energy conservation is not maximized by the consumption of smaller prey (Butler, 1988). When given a choice between active prey in different size categories, *L. macrochirus* pursue what is perceived to be the larger prey item. Chosen prey may actually be larger or more proximal than other prey, thus giving the appearance of greater size (Kao, 1985).

As *L. microchirus* age, visual resolution as well as consumption rates increase (Kao, 1985). Better vision enables fish to forage more efficiently but the costs of searching and handling prey increase. Changes in vision and metabolism lead to an increase in mean prey size and a movement from littoral zones to open water.

Research Hypothesis And Objectives

It has been hypothesized that a linkage may exist between exposure to copper and predation. The purpose for water quality criteria established by the EPA is to protect organisms from acute effects (such as mortality) and chronic effects (such as growth and reproduction) of point source pollution. However, it has become apparent that

amphibians may experience negative behavioral and physiological effects from sublethal concentrations of heavy metals polluting aquatic environments.

Copper is a naturally occurring element, but most copper found in aquatic systems is from anthropogenic sources such as wastewater effluent and application of herbicides to aquatic habitats (ATSDR, 1990). Although previous studies indicate *R. pipiens* display tail kinking and erratic swimming at copper concentrations as low as 0.04 mg/L (Gottschalk, 1995), this concentration is commonly exceeded in surface waters of the northern United States (Lopez, 1977). Throughout the Great Lakes area, copper contamination (Great Lakes Water Quality Board, 1995) as well as the declining *R. pipiens* population throughout the northern United States continues to be of primary concern. Several studies on the ecotoxicology of copper allude to increased predation being a result of contamination, but rarely are experiments carried out to determine possible ecological ramifications to sublethal concentrations of copper. In an effort to address the lack of research involving sublethal effects of copper on predation rates, the following objectives are proposed in this study:

1. Establish aqueous Cu concentrations that will not cause direct mortality in larval *R. pipiens;*
2. Duplicate morphological effects of Cu exposure observed in previous studies (Gottschalk, 1995);
3. Determine if tadpole exposure to sublethal Cu concentrations will cause an inability to detect the presence of a predator, thereby increasing predation;
4. Determine if exposure to sublethal Cu concentrations will physically impair tadpoles to the point where escaping predation is impossible; and

5. Understand how sublethal exposure leads to a concentration dependant recovery regarding body length.

CHAPTER 3

MATERIALS AND METHODS

This chapter provides a discussion of methods, materials, experimental design, and analytical and statistical tests used in this study. Specific topics to be addressed here include:

A. Tadpole culturing methods;
B. Methods to establish water quality;
C. Experiments to determine sublethal range;
D. Sublethal exposure;
E. Fish culturing methods;
F. Methods for predaton experiments;
G. Methods for growth recovery experiment; and
H. Analytical techniques;

Tadpole Culturing Methods

Rana pipiens eggs were purchased from Carolina Biological Supply. Upon arrival, the eggs were transferred to a high-density polyurethane container and allowed to acclimate to room temperature. Hardness of the original water was measured so embryos could be properly acclimated to dechlorinated tap water at 20º C in the laboratory. Dechlorinated tap water was softer (104 total hardness as $CaCO^3$) than the water in which the tadpoles arrived (156 total hardness as $CaCO^3$) so 500 ml dechlorinated tap water was added twice daily until toxicity testing began (about 7 days). A 16:8 full spectrum light dark cycle was maintained throughout the experiment. Water was aerated gently to avoid disturbing the egg clutch.

Water Quality Parameters

Dechlorinated tap water was stored in a 384 L. (100 gal) high-density polyurethane container. All water used throughout the experiment came from this container to sustain consistent water quality properties. Metal bioavailability changes as water quality parameters change; therefore it is important to understand various solvent characteristics. Water parameters controlled for included: dissolved oxygen (DO), conductivity, pH, hardness, and alkalinity. Water quality characteristics were measured at the beginning of and during the experiment. DO and conductivity were measured using the YSI model 85 probe. An Orion model SA520 probe was used to measure pH. Hardness and alkalinity were measured following titration methods from *Standard Methods for the Examination of Water and Wastewater*. Both instruments were calibrated according to manufacturer guidelines.

Range Finding Experiments

Serial dilutions were mixed using $CuNO_3$ diluted with dechlorinated tap water from the storage tank. These premixed stock solutions were used during the toxicity experiment. After hatching, tadpoles matured to 4 days of age, at which time a 96-hour LC50 was conducted to establish a range of sublethal concentrations. A 16:8 full spectrum light dark cycle was maintained. Mason jars cut in half served as exposure chambers with each containing 300 ml of solution. Copper nitrate was diluted to 0.003, 0.03, 0.3, 3, 30, and 300 mg/L Cu. Three replicates of five tadpoles were randomly selected from the container using a turkey baster and placed into a concentration or control. Identical methods were performed using $CuNO_3$ diluted to concentrations of

0.019, 0.0375, 0.075, 0.15, 0.3 mg/L Cu. Tadpoles were monitored at 12-hour increments to gauge toxic response. The null hypothesis for this portion of the experiments is as follows:

Ho: There is no significant difference in mortality between control tadpoles and those exposed to copper nitrate concentrations.

Seven Day Sublethal Toxicity

The range finding experiments indicate tadpoles are affected by Cu concentrations between 0.14 mg/L and 0.02 mg/L. Replicates of 10 *Rana* larvae were chosen randomly and exposed to one of 5 Cu concentrations (0.1, 0.08, 0.06, 0.04, 0.02, mg/L, respectively) plus controls. Cu concentrations were analyzed at the beginning and end of experiment. Tadpoles were exposed for 7 days and fed crushed alfalfa pellets every other day 2 hours before the solution was changed. The null hypothesis for this portion of the experiment is as follows:

Ho: There is no significant observable behavioral difference between control tadpoles and those exposed to concentrations of copper nitrate.

Fish Culturing Methods

Thirty-four *L. macrochirus* of lengths ranging from from 10-14 cm were provided by The Lake Doctor, a fishery located in Celina, TX. The fish were housed together in

one 230 L aquarium filled with dechlorinated tap water. Fish were fed generic cichlid flakes and pellets once daily. Tadpoles not needed for the toxicity experiments were fed to fish daily to ensure fish would recognize tadpoles as a prey item. A 16:8 full spectrum light dark cycle was maintained. Forty-eight hours prior to initiation of predation experiments, food was withheld from the fish.

Predation Experimental Methods

For the predation experiments, two 19 L aquaria were placed side by side, surrounded by black plastic to avoid distracting fish or tadpoles. Water temperature was maintained at 20°C with a 16:8 light dark cycle throughout the experiment. On day 11, two *L. macrochirus* were selected using a net, from the large aquarium and placed in a smaller chamber within the tanks, devoid of food, to acclimate to water containing prey. Tadpoles taken from the toxicity study were placed into a tank one replicate at a time. After the tadpoles were transferred on day 11, initial swimming/hiding behavior was observed. Thirty minutes later, *L. macrochirus* were released from holding chambers and predation behavior recorded by video camera. After the fish consumed all the tadpoles in the tank, or after 60 minutes had passed, fish and remaining tadpoles were removed. It is important to note, numbers of tadpoles in each predation replicate changed depending on the mortality occurring throughout the toxicity experiment. Higher copper concentrations resulted in higher tadpole mortality, therefore fewer tadpoles exposed to higher concentrations were available for the predation experiment. Predation rates (tadpoles consumed / minute) were used in data analysis to compensate for differences in available prey. Fish that had previously been used in a feeding trial were temporarily housed in a

separate location until the end of the predation experiment to ensure fish involved in additional replicates were hungry. The null hypothesis for this portion of the experiment is as follows:

Ho: There is no significant difference in predation rates between control tadpoles and those exposed to concentrations of copper nitrate.

Growth Recovery Experiment

After the toxicity portion of the study, it was apparent tadpoles exposed to higher sublethal concentrations were smaller than controls. An experiment to monitor growth and possible recovery of tadpoles exposed to copper was performed. After the toxicity test, a portion of tadpoles exposed to 0, 0.04, 0.08, and 0.1 nominal concentrations were maintained until the appearance of front leg buds to record growth after exposure. Tadpoles utilized in the growth recovery experiment were not used in the predation experiments. Eight 38 L aquaria, each divided into four equal compartments using polyurethane screen and filled with dechlorinated tap water housed the tadpoles. Tadpoles from the same clutch were housed in the same aquaria ensuring identical age. Water temperature was maintained at 20°C using pre set water heaters with a 16:8 light dark cycle throughout the experiment. Tadpoles were fed crushed alfalfa pellets. Waste was siphoned off bottom and water changed weekly. Tadpoles were transferred to a flat-bottomed specimen jar and length was measured weekly using graph paper. The null hypothesis tested for this portion of the experiment is as follows:

Ho: There is no significant size difference between control tadpoles and those exposed to concentrations of copper nitrate.

Analysis

Solution samples were taken at toxicity test initiation and termination. Samples were held in 100 ml Nalgene bottles and acidified to a pH < 2 with nitric acid. Metal concentrations were later analyzed by flame atomic absorption spectroscopy using a Perkin-Elmer 2380 spectrophotometer. Wavelength was 324.8 nm and slit band width was 0.7 nm as determined by the Perkin-Elmer manual.

Toxicity data were analyzed using Probit analysis using Toxstat (West inc. and Gully 1994) to determine LC 50 concentrations. Predation data were analyzed by ANOVA and grouped by SNK procedures using SAS (SAS Institute Inc. 1989). Growth recovery data were found to be non-normal and analyzed by Kruskal Wallis procedures using SAS (SAS Institute Inc. 1989). Results from ANOVA and Kruskal Wallis analysis can be found in appendix 1.

CHAPTER 4

RESULTS

Several linked experiments were performed in this study to better understand the ecological implications sublethal copper (Cu) concentrations may have in regard to predation and growth. The toxicology experiments were designed to elicit information on Cu concentrations that do not result in tadpole mortality but do cause aberrant behavior. Predation experiments were then performed on tadpoles to determine the effects of sublethal Cu exposures on predator prey relationships. Lastly, to determine effects Cu exposure has on growth after exposure, tadpoles were removed from treatment solutions and measured weekly. Reported in this chapter are results to the following experiments:

- Water quality parameters**;**
- 96-hour LC50;
- 7-day toxic response;
- Aberrant behavioral responses;
- Predation rates;
- Growth recovery response;

Water Quality Parameters

Depending on water quality, Cu may become more or less available to tadpoles. To ensure consistency in Cu bioavailability throughout the experiments, characteristics of dechlorinated water were measured before and during all three experiments. Standard water quality characteristics included: dissolved oxygen (DO), hardness, alkalinity, conductivity, and pH.

Water quality parameters did not differ from test initiation to termination or between experiments. The ranges of water quality values measured throughout experiments are as follows: pH (7.35-7.88), alkalinity (46-57 mg/L $CaCo_3$), DO (7.2-8.8), hardness (96-106 mg/L $CaCo_3$) and conductivity (510-550 μmhos/cm). Water quality parameters did not differ after addition of copper.

Initial Toxicity Response

The 96-hour toxicity tests were designed to determine the upper Cu concentration limits for tadpoles during a 7-day sublethal exposure. Table 1 displays LC50 toxicity data as determined by probit analysis for three toxicity tests. After 96-hour exposure, the LC50 for tadpoles exposed to Cu was 0.083 mg/L with the 95% confidence limits being 0.067-0.089 mg/L for the first replicate. The second toxicity test indicated a LC50 of 0.092 mg/L with the 95% confidence interval 0.063 – 0.105 mg/L. An LC50 of 0.95 mg/L with 95% confidence interval 0.077-0.114 mg/L was determined after the third 96-hour test.

Results of the 96-hour toxicity test are slightly different than what is generally reported by other researchers. The values generated for this experiment are higher than those typically reported; although all reported LC50s do fall within the 95% confidence levels calculated for these experiments (Gottschalk, 1995). It is important to note, water quality characteristics measured in these experiments differ from those reported in Gottschalk's 1995 study.

7-Day Toxicity Response

The LC50 calculated for a 7-d exposure to Cu was 0.074 mg/L with the 95% confidence interval 0.067-0.082 mg/L for the first experiment. For the second 7-d exposure the LC50 was 0.072 mg/L Cu and the 95% confidence interval was 0.063 – 0.079 mg/L Cu. An LC50 of 0.67 mg/L, and 95% confidence interval 0.055-0.084 mg/L was calculated for the third exposure. Literature searches of databases including CARL and Ecotox revealed no 7-day exposure information on amphibians.

Figure 1 indicates a toxic response at concentrations as low as 0.036 mg/L. Figures 1-3 depict three separate toxicity tests that indicate a clear increase in mortality as a function of concentration as well as exposure time. Figures 1-3 also indicate that the most dramatic lethality occurred in concentrations above 0.07 mg/L Cu in the first 48 hours of exposure. After the first 48 hours, the mortality response becomes more gradual which is a fairly common toxic response.

Table 1

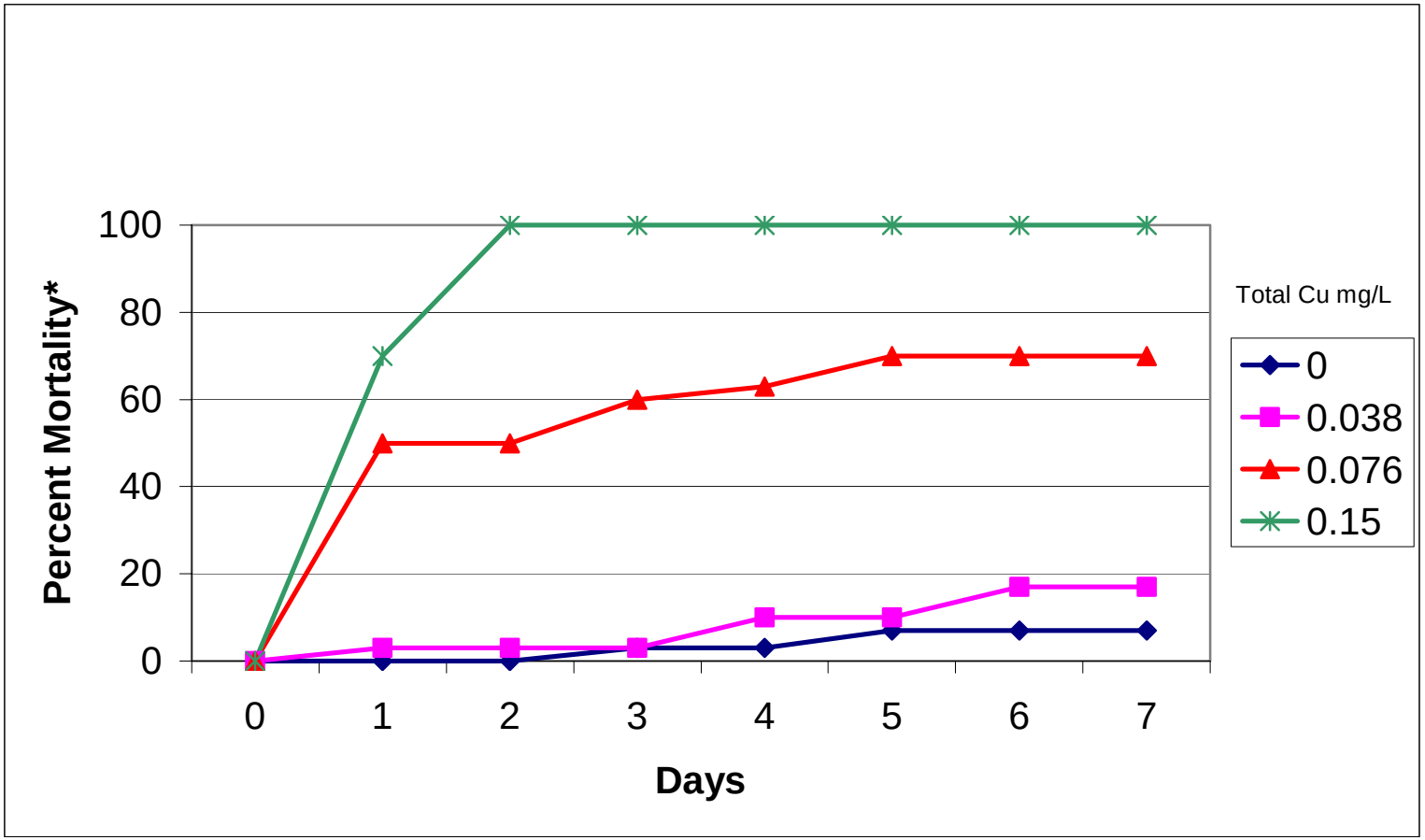
Toxicity Data from Tests of Varying Lengths

Test Duration	LC50	Lower 95%	Upper 95%	n
96-hour	0.083	0.067	0.089	90
96-hour	0.092	0.063	0.079	90
96-hour	0.095	0.077	0.114	90
7-day	0.074	0.065	0.082	120
7-day	0.072	0.063	0.079	150
7-day	0.078	0.055	0.084	180

LC50 and confidence intervals calculated by Probit analysis using Toxtat.

Figure 1

Cumulative Mortality Response of *R. pipiens* Exposed to Copper** mg/L (Rep 1)

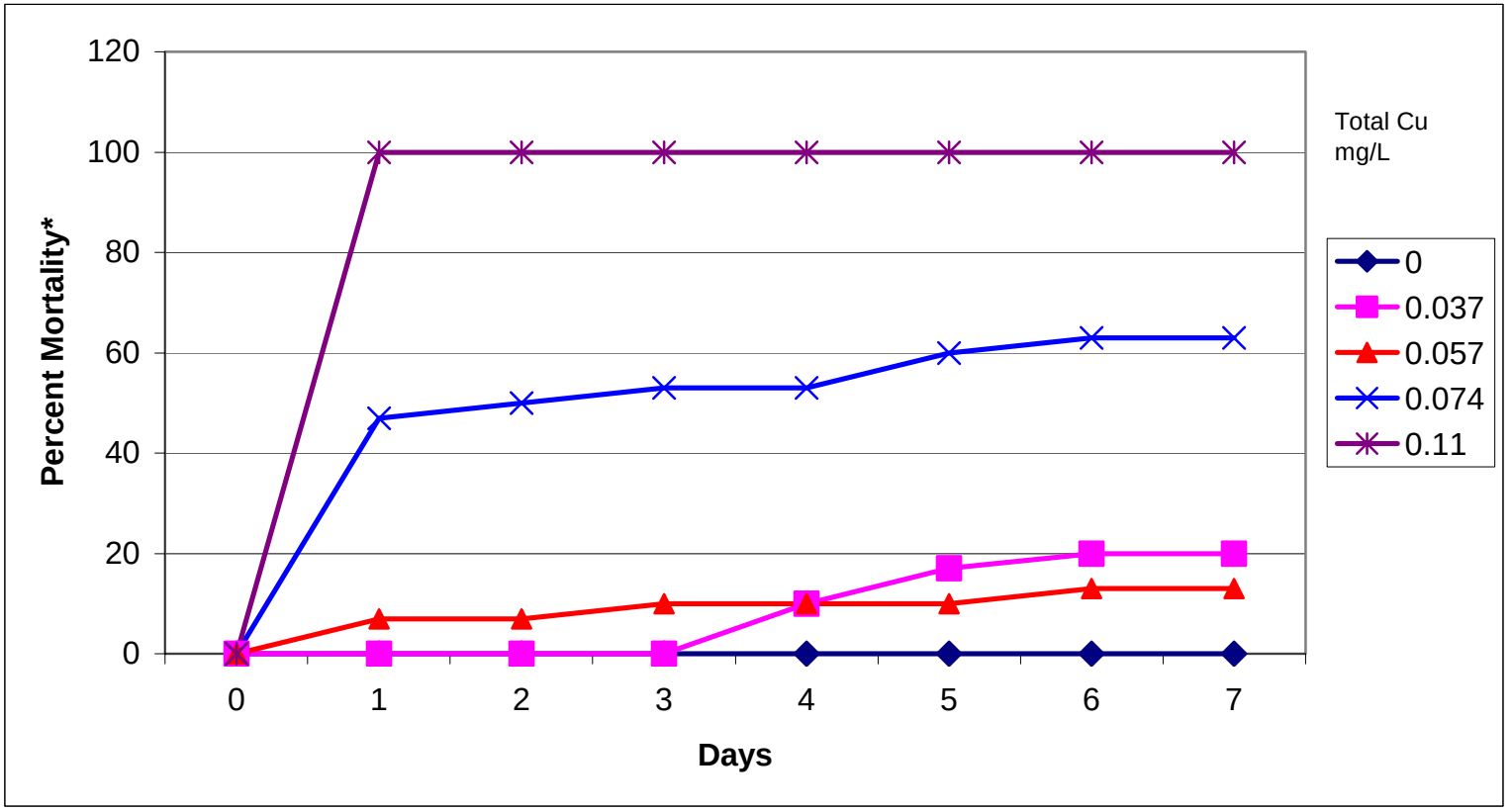


*n = 30 for all concentrations

** Cu as $CuNO_3$

Figure 2

Cumulative Mortality of *R. pipiens* Exposed to Copper** mg/L (Rep 2)

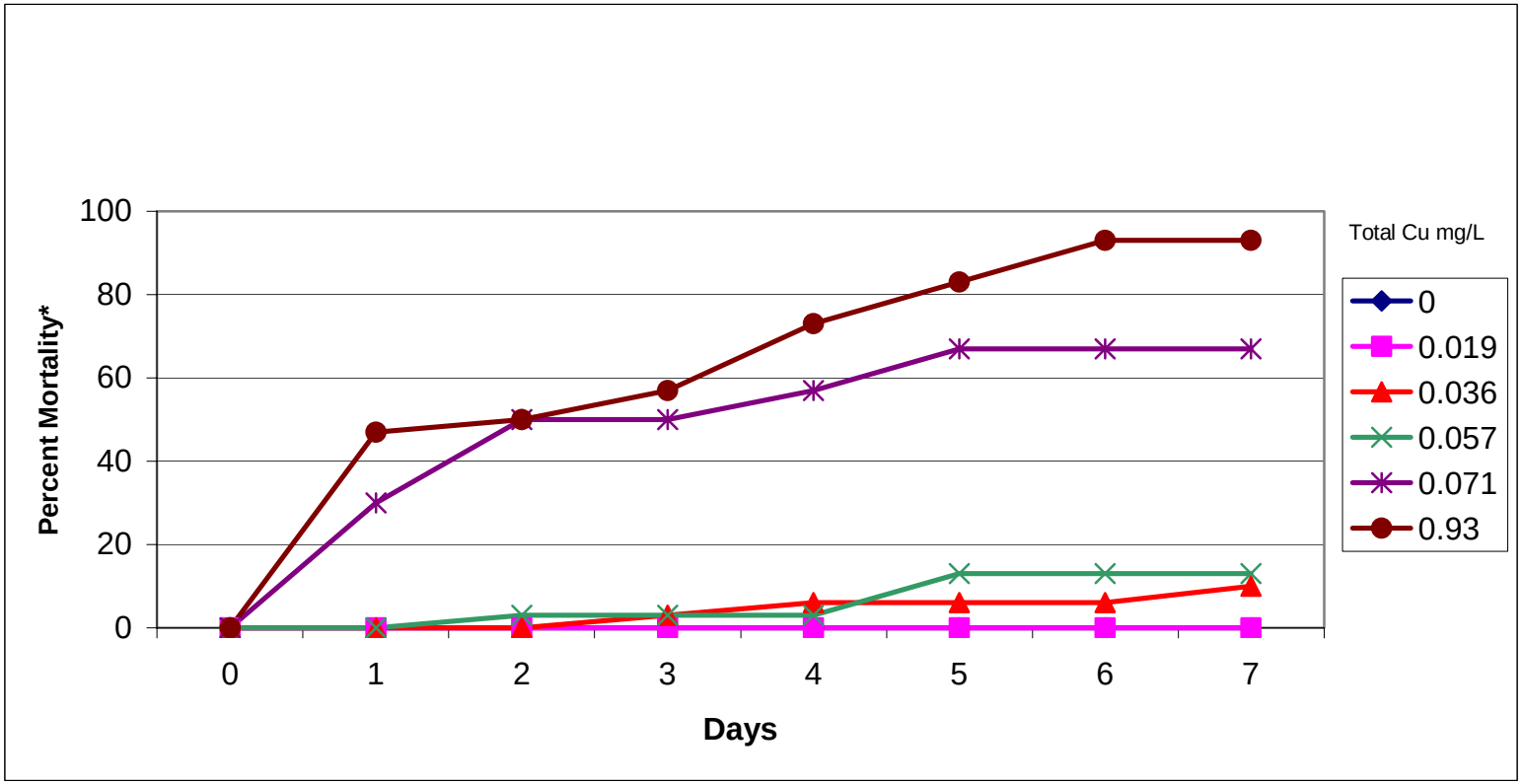


* N = 30 tadpoles for each concentration

** Cu as $CuNO_3$

Figure 3

Cumulative Mortality Response of *R. pipiens* Exposed to Copper** mg/L (Rep 3)



* N = 30 tadpoles for each concentration

** Cu as $CuNO_3$

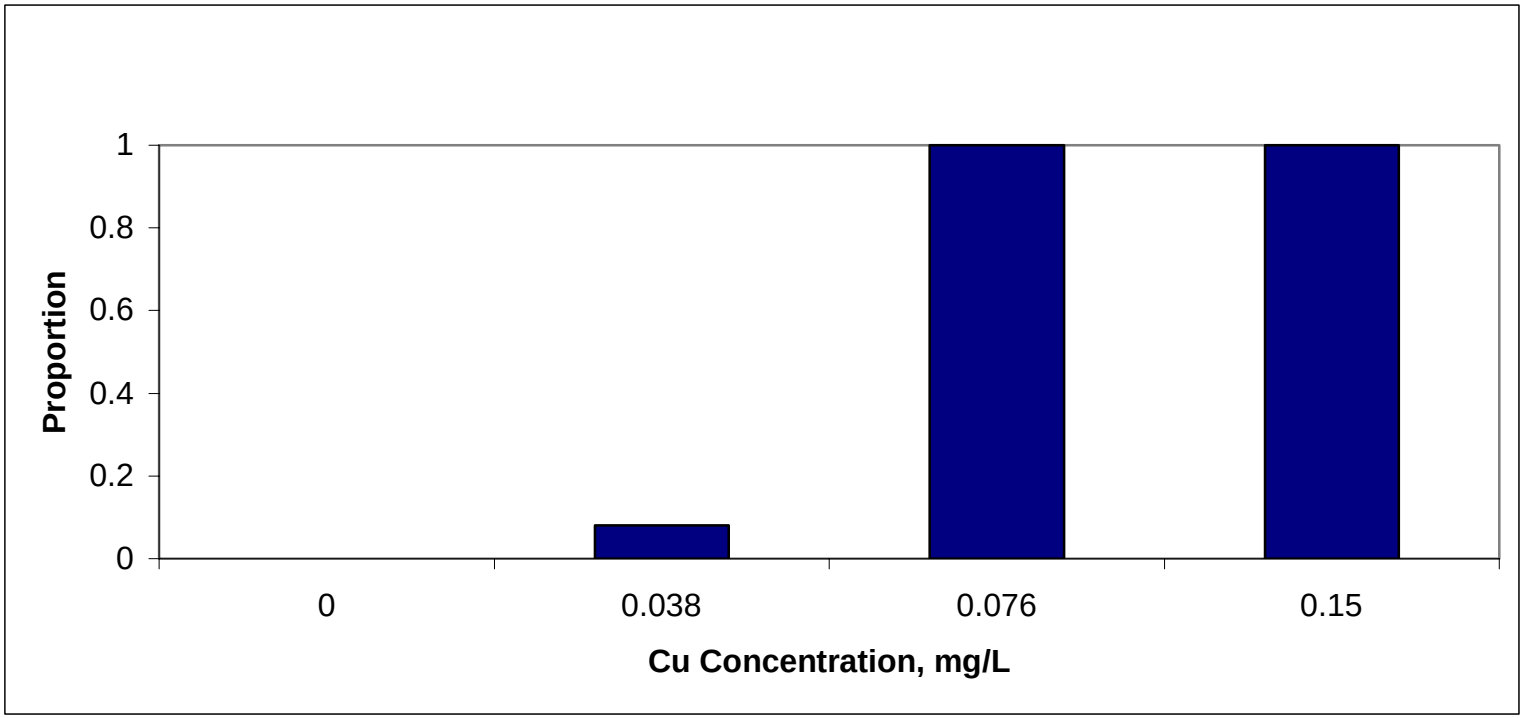
Aberrant Behavioral Response

Figures 3-6 depict Cu concentrations at which aberrant behavior becomes apparent. Aberrant behavior is defined as sporadic, twitchy, or struggled movement. When disturbed, impaired tadpoles would attempt to swim, but achieved only sporadic twitching in place. Undisturbed tadpoles lay at the bottom of the exposure chamber on a side or upside down, moving very little.

Copper was observed to induce aberrant behavior at concentrations as low as 0.036 mg/L (Figure 5), which is nearly 50% lower than the calculated LC50 for a 7-d period. At Cu concentrations above 0.071 mg/L, all tadpoles exhibited struggled or sporadic movement. Tadpoles exposed to concentrations above the LC50 displayed aberrant behavior sooner and more dramatically than did tadpoles exposed to concentrations lower than the LC50. After aberrant behavior was apparent, the response continued until organisms were removed from solution or mortality occurred. Affected tadpoles appeared to display normal behavior within one week after transition to uncontaminated solution.

Figure 4

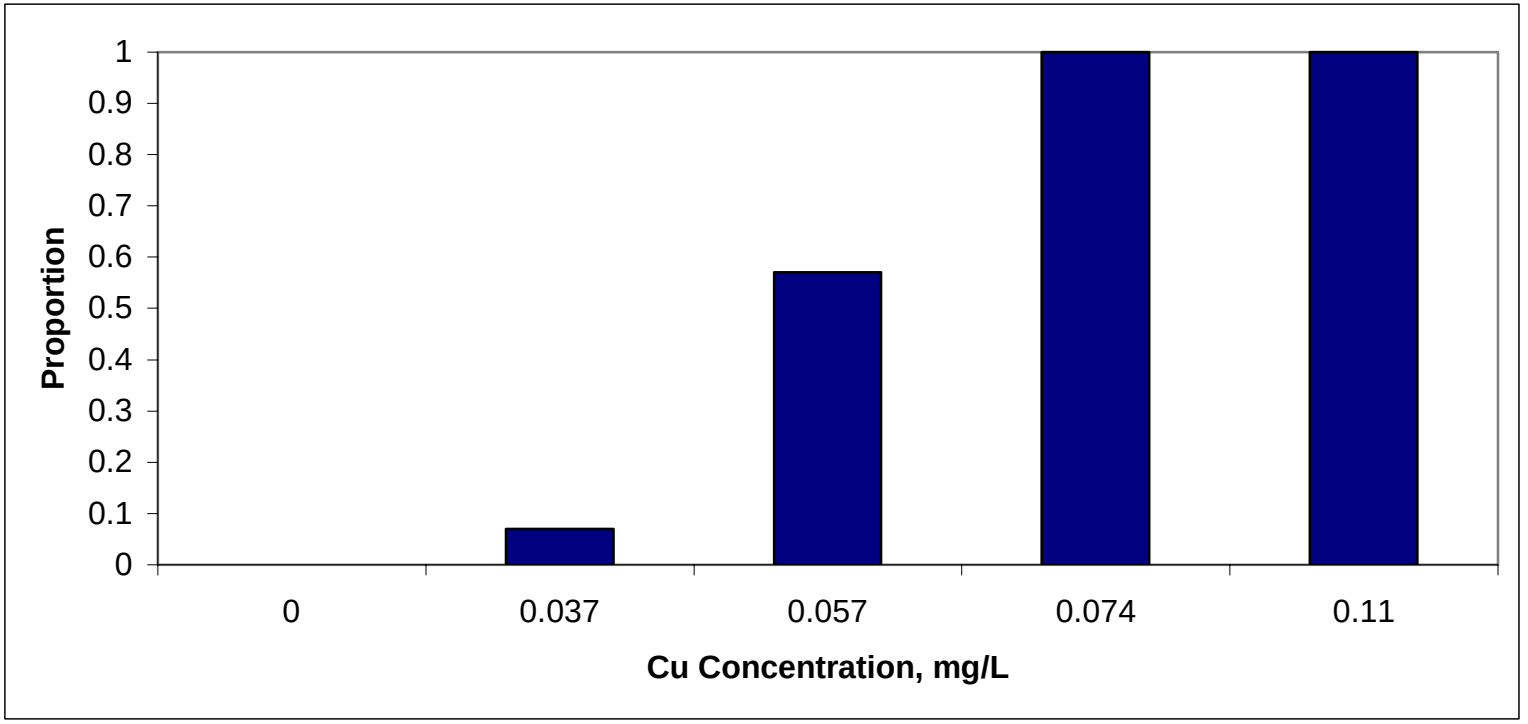
Proportion Of Tadpoles Displaying Aberrant Behavior*
At Different Cu Concentrations (Rep 1)



*Aberrant behavior is defined as sporadic, twitchy, or struggled movement.
n = 30 for all concentrations

Figure 5

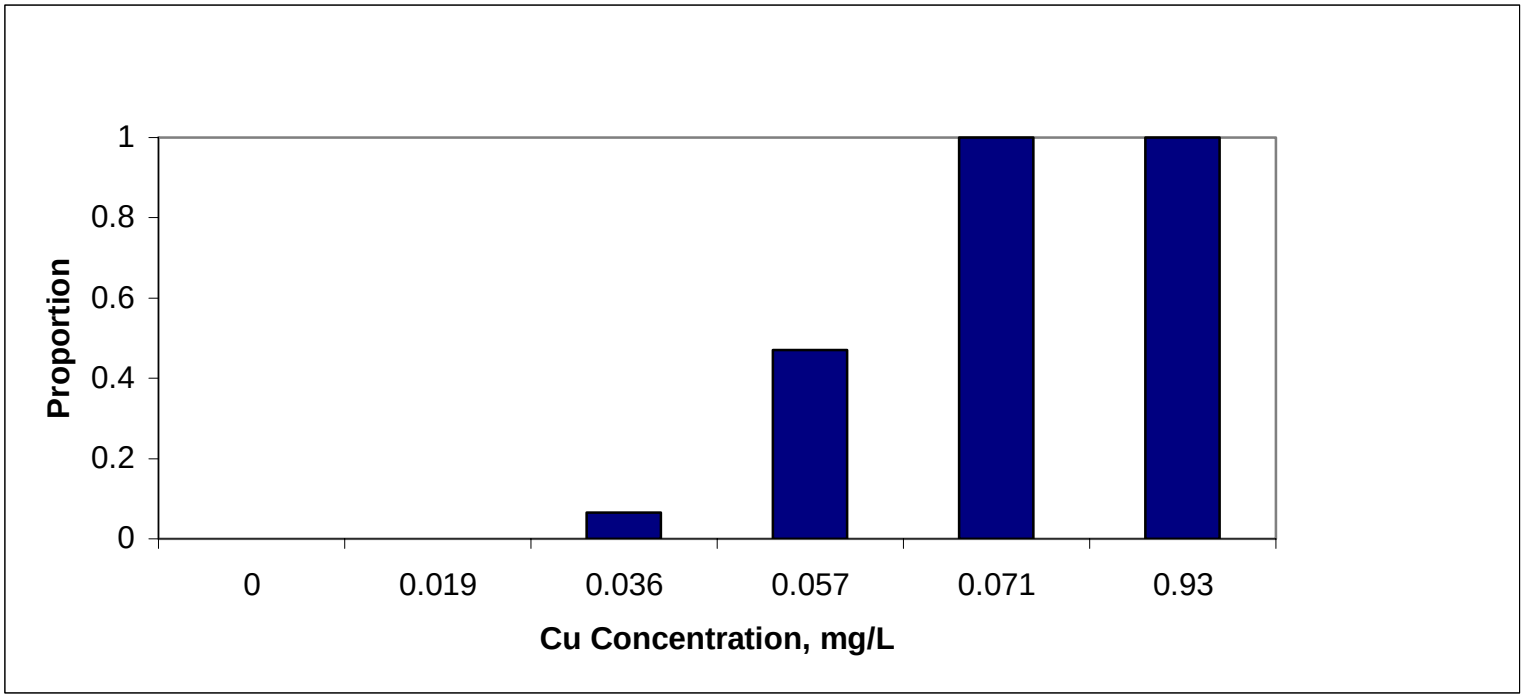
Proportion of Tadpoles Displaying Aberrant Behavior*
at Different Cu Concentrations (Rep 2)



*Aberrant behavior is defined as sporadic, twitchy, or struggled movement.
n = 30 for all concentrations

Figure 6

Proportion of Tadpoles Displaying Aberrant Behavior*
at Different Cu Concentrations (Rep 3)



*Aberrant behavior is defined as sporadic, twitchy, or struggled movement.
n = 30 for all concentrations

Predation Data

The number of tadpoles surviving the toxicity portion of the experiment varied, depending on exposure concentrations. Table 2 summarizes the number of tadpoles available for each predation experiment and replicate. Predation data indicate a significant difference in predation rate between control groups and the highest concentration at α=0.05. Predation experiments 2 and 3 indicate a significant difference between the control group and the 2 highest concentrations (ANOVA p=0.002 p=0.04 respectively) (Table 3). Predation experiment 1 indicates a significant difference only in the highest concentration (ANOVA p= 0.04). Figures 7-9 illustrate tadpoles consumed per minute at different concentrations of copper exposure.

Table 2 Number of Tadpoles available for Consumption After Toxicity Testing

Concentration mg/L	Replicate 1	Replicate 2	Replicate 3	Replicate 4
0	10	10	10	10
0.038	9	9	10	9
0.074	6	7	6	5
0.14	0	0	0	0
0	10	10	10	10
0.037	10	9	10	10
0.057	9	8	8	10
0.074	7	4	6	7
0.11	2	3	1	3
0	9	10	10	10
0.019	10	10	10	10
0.036	9	10	9	10
0.057	10	8	9	9
0.071	8	8	8	7
0.093	3	5	4	4

Table 3 Concentrations Indicating Predation Rates Significantly Different than Controls

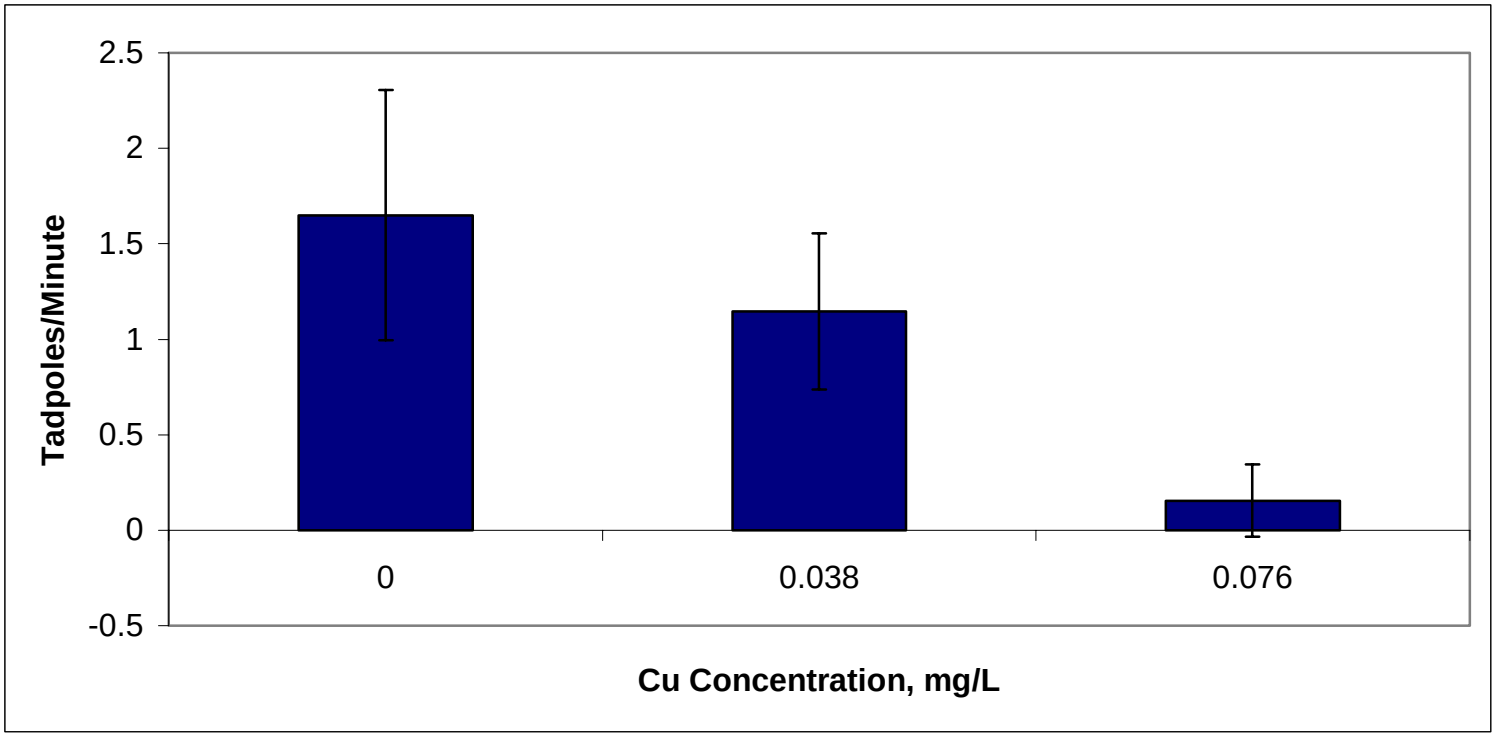
Predation experiment	*n*	*P Value*	Concentrations Significantly Different than Controls
1	24	0.04	0.074
2	20	0.04	0.074, 0.11
3	36	0.002	0.071, 0.093

Significance determined by ANOVA $\alpha = 0.05$

It was hypothesized that predation of tadpoles exposed to copper would occur at a significantly higher rate than unexposed tadpoles, which would indicate either a lack of predator detection or impaired escape behavior. Although a significant difference in predation rate was suggested, predation rates of exposed tadpoles were significantly lower than predation rates of controls. Intuitively, if tadpoles fail to display escape behavior, predation rate should increase. However, predator behavior is critical and cannot be neglected.

Figure 7

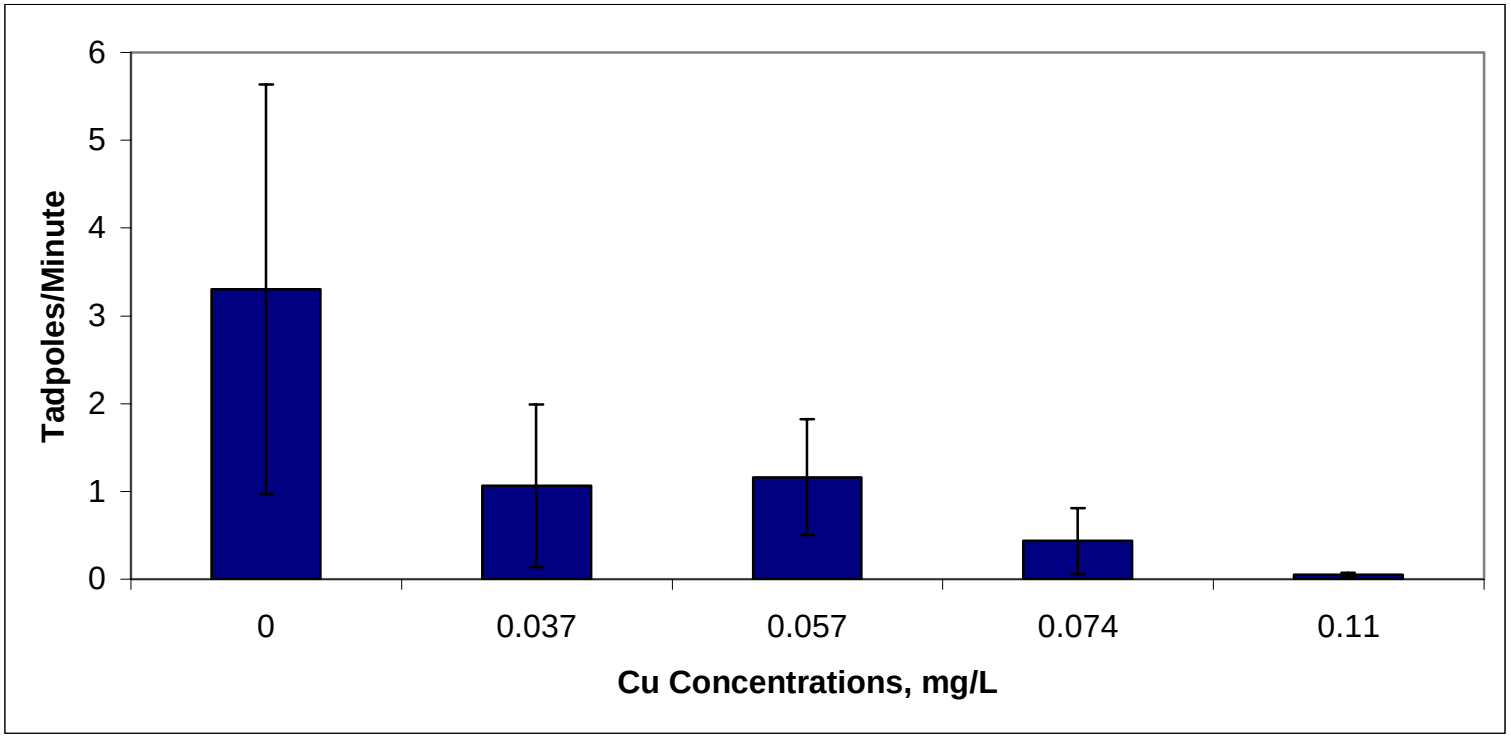
Predation Rate* of *R. pipiens* Tadpoles by *L. macrochirus*
After Copper Exposure (Rep 1)



* Tadpoles consumed per minute = predation rate

Figure 8

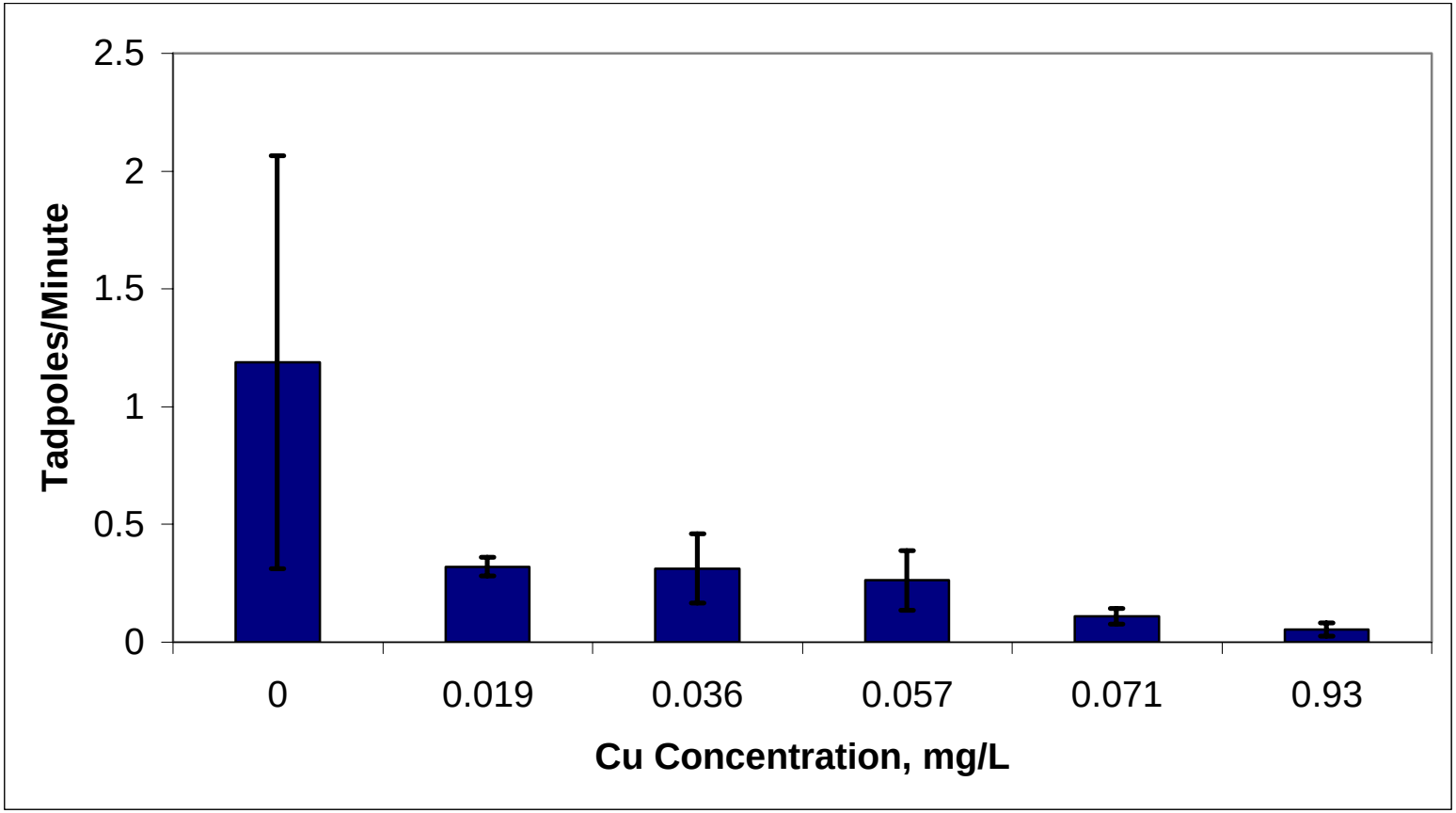
Predation Rate* of *R. pipiens* Tadpoles by *L. macrochirus*
After Copper Exposure (Rep 2)



* Tadpoles consumed per minute = predation rate

Figure 9

Predation Rate* of *R. pipiens* Tadpoles by *L. macrochirus*
After Copper Exposure (Rep 3)



* Tadpoles consumed per minute = predation rate

Growth Response

This portion of the experiment was designed to determine if tadpoles recover from Cu exposure stress in terms of body length prior to metamorphosis. After the sublethal exposures, tadpole size in different Cu concentrations was observably different. Stress recovery experiments have been performed using fish (Reddy, 1998) but not amphibians.

There was no difference in tadpole body lengths between the control groups and concentrations below 0.038 mg/L ($p=0.16$ Kruskal Wallis). Copper concentrations above 0.071 mg/L significantly hindered growth in exposed tadpoles at $\alpha=0.05$ ($p = .0001$ Kruskal Wallis) directly after toxicity experiment. On day 19 (8 days after transfer to uncontaminated solution) a significant difference ($p=0.0051$ Kruskal Wallis) existed between controls and tadpoles exposed to Cu concentrations above 0.071 mg/L. On day 26 (15 days after transfer to uncontaminated solution) a significant difference ($p=0.043$ Kruskal Wallis) existed for tadpoles exposed Cu concentrations above 0.071 mg/L. In all three replicates, a significant relationship ($p>0.24$ Kruskal Wallis) between exposure concentration and length failed to exist beyond 30 days of age (19 days after tadpoles were removed from solution).

Figures 10-12 depict length over time until the front leg buds were visible. As exposed tadpoles recovered in terms of body length prior to metamorphosis, I assume they would have been of normal size after metamorphosis.

Figure 10

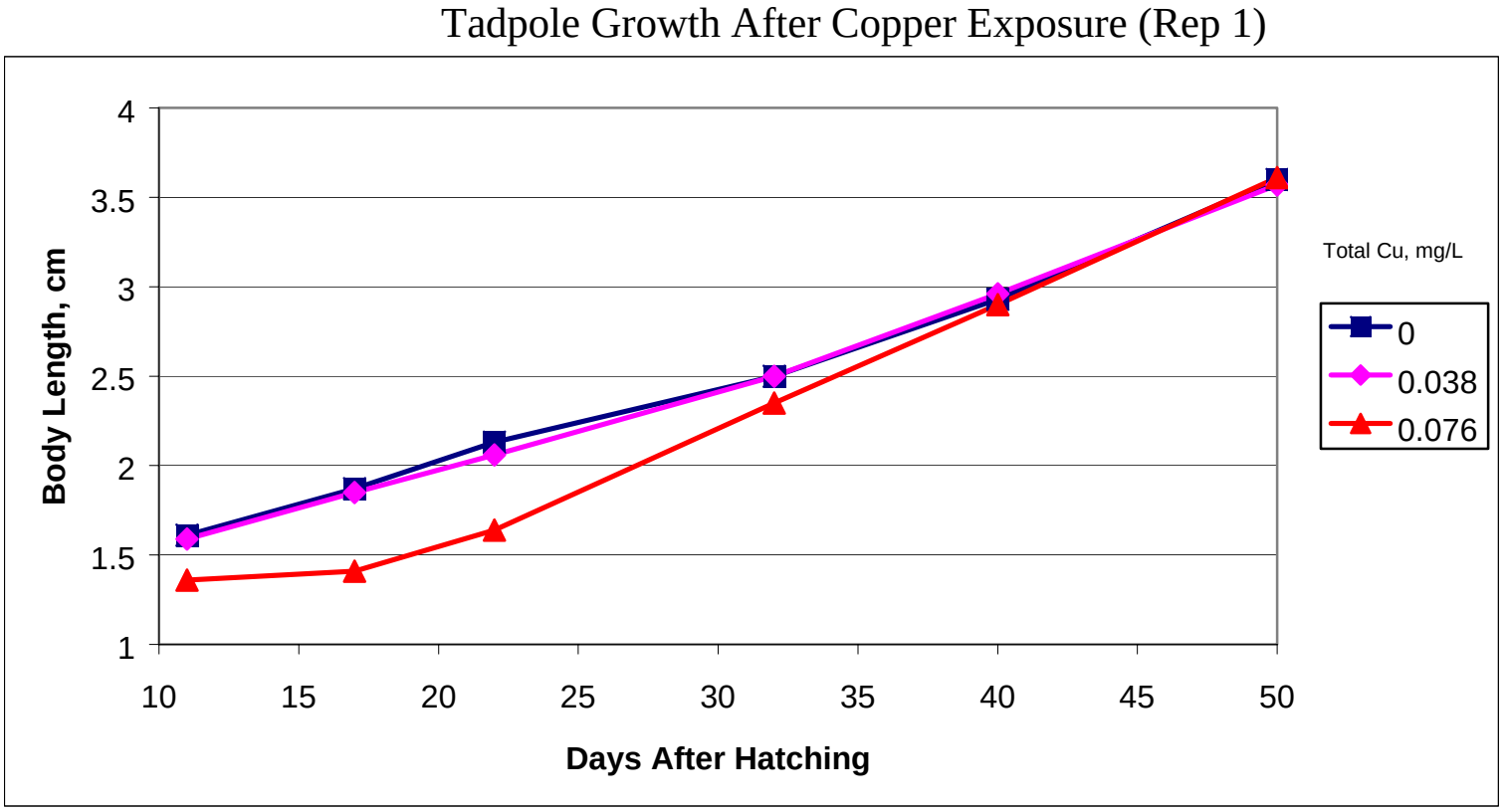

Tadpole Growth After Copper Exposure (Rep 1)
Body Length, cm
4
3.5
3
2.5
2
1.5
1
10
15
20
25
30
35
40
45
50
Days After Hatching
Total Cu, mg/L
0
0.038
0.076

Figure 11

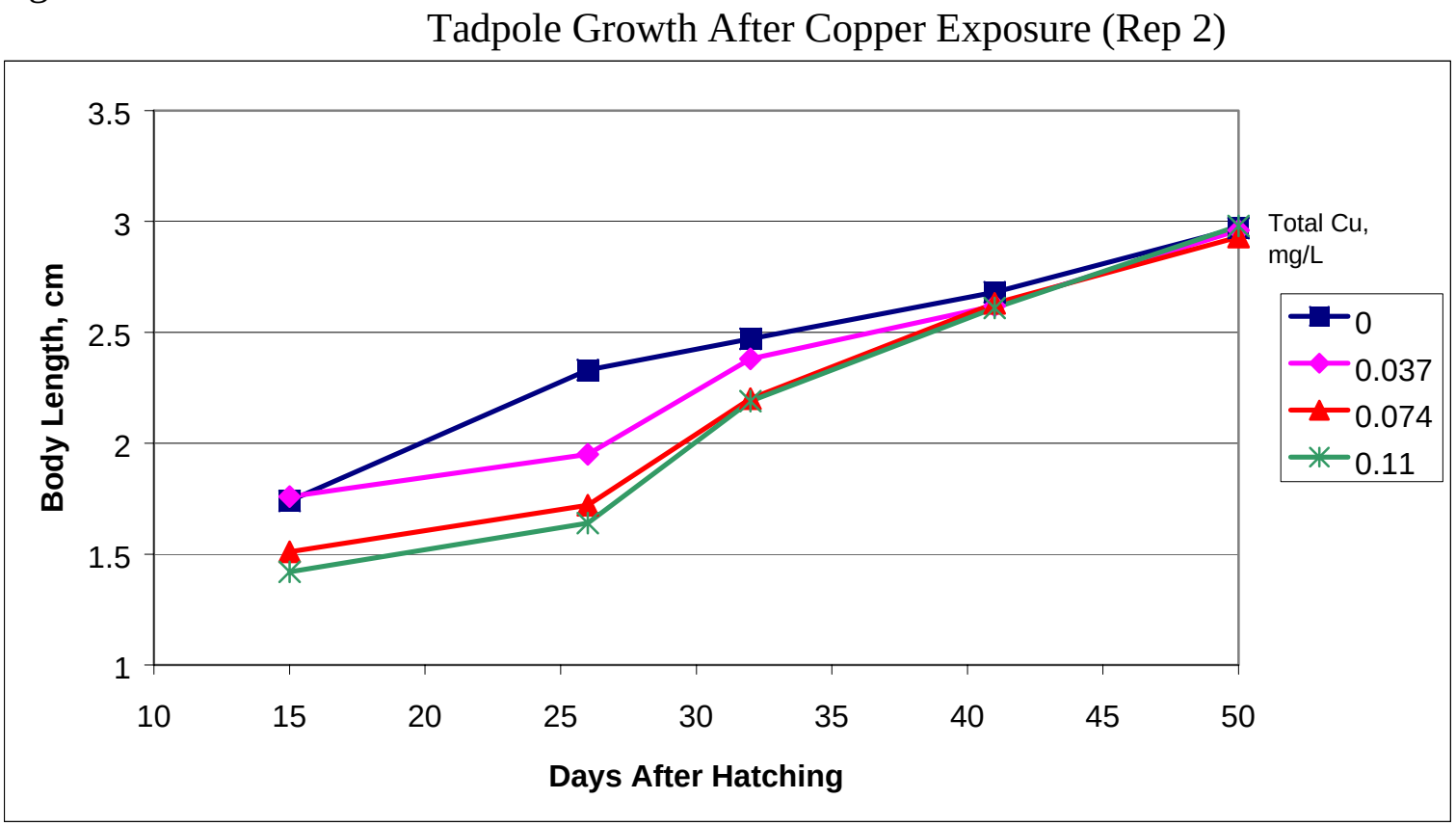
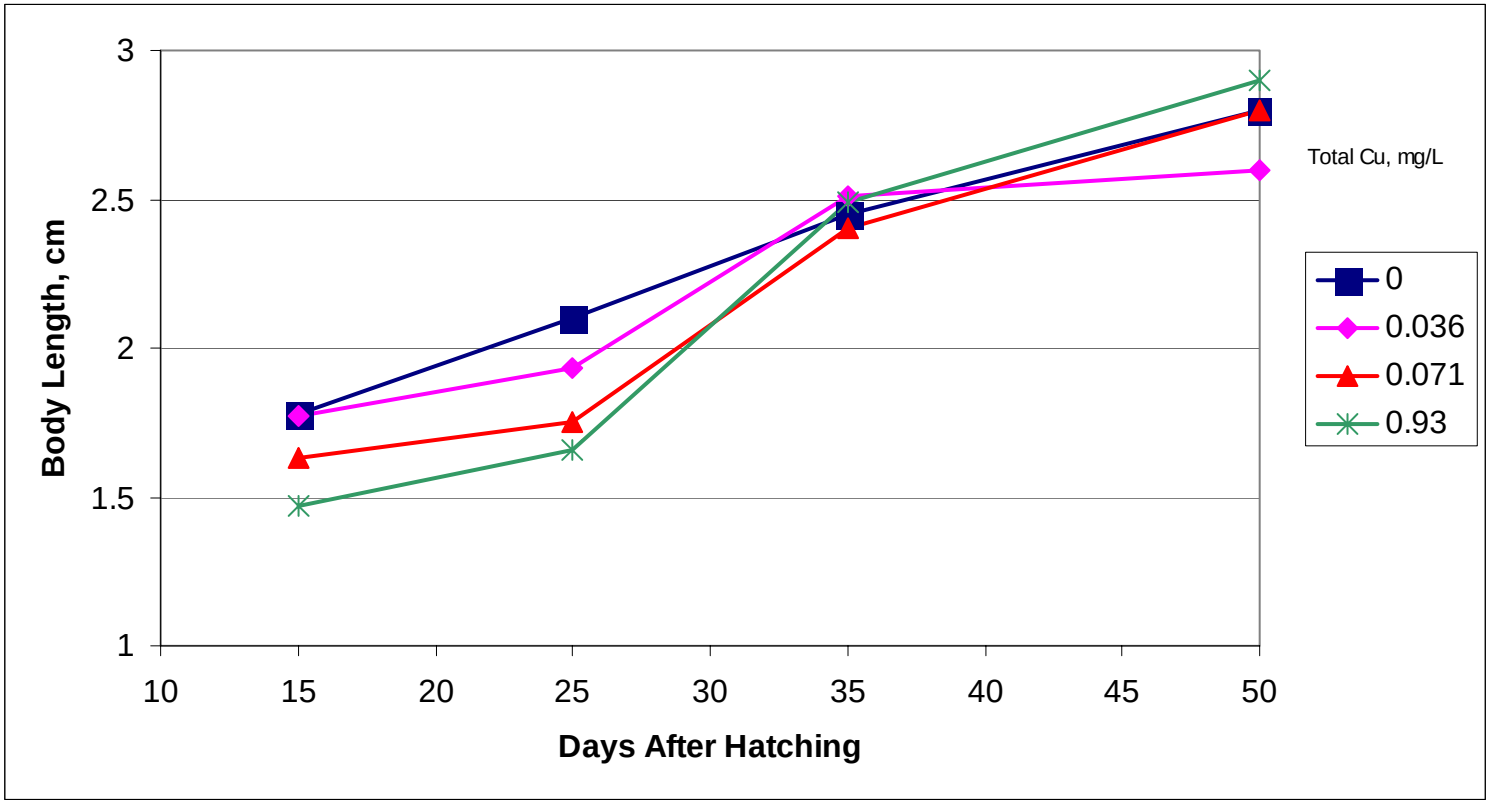

Tadpole Growth After Copper Exposure (Rep 2)
Body Length, cm
3.5
3
2.5
2
1.5
1
10
15
20
25
30
35
40
45
50
Days After Hatching
Total Cu, mg/L
0
0.037
0.074
0.11

Figure 12

Tadpole Growth After Copper Exposure (Rep 3)



CHAPTER 5

CONCLUSIONS

The primary objectives of this thesis were to determine the effects of sublethal Cu exposure on the ability of *R. pipiens* tadpoles to (1) escape predation and (2) recover with respect to body length prior to metamorphosis. To satisfy these objectives an LC50 concentration was determined for *R. pipiens*. LC50 concentrations established the high concentrations for the longer 7-d exposure designed to reveal behavioral or morphological differences, but without causing mortality. After 7-d exposures, tadpoles were either subjected to predation or reared in a tank to monitor body length until the appearance of frontal leg buds.

It is important to note this was a laboratory experiment and the intent was not to duplicate a natural environment. A natural pond or lake environment usually contains humic and fulvic acids from decaying plant vegetation that would bind cupric ions. Sediments also act as a sink for Cu in a natural setting. In aquatic ecosystems Cu binds to sediments and organic matter, which reduces cupric ion activity, thereby reducing toxic effects. However, excess Cu is still cause for concern in many water bodies.

Cu is considered a priority pollutant and is regulated by the Environmental Protection Agency. The acute maximum amount of total Cu allowed in a freshwater system is 0.013 mg/L (hardness 165 mg/L as $CaCO_3$)(US EPA, 1998), which is well below the no observable effects level for this experiment. However, this concentration

is commonly exceeded in surface waters of the northern United States (Lopez, 1977). Total Cu concentrations range from 0.0003 to 9 mg/L in areas where localized anthropogenic inputs occur (Leland, 1985). Throughout the Great Lakes area, Cu contamination (Great Lakes Water Quality Board, 1995) as well as declining *R. pipiens* populations continue to be of primary concern.

Niche specificity tends to be positively correlated with contaminant sensitivity and negatively correlated with genetic diversity (Birge, 1979). Anuran species that have adapted to a single habitat are less likely to have adaptive mechanisms that would enable them to acclimate to environmental changes. Range of *R. pipiens* extends from the east coast to the west coast and from the mid United States to Canada. *R. pipiens* tadpoles are found in various habitats from brackish waters to pristine mountain lakes.

R. pipiens in different environments have different genetic characteristics that allow them to adapt. The *pipiens* species has at least two genetic morphs that are distinguishable by color and habitat. The mottled "kandiyohi" morph breeds in prairie ponds subject to desiccation. Tadpoles with this color pattern metamorph into adults in a shorter time span than either the normal colored tadpole or spotted morph. For reasons unknown, the spotted "burnsi" morph has a higher survival rate in Northern United States and Southern Canada where winters are extremely harsh (Duellman,1986). Genetic diversity may also influence contaminant sensitivity between anuran species and subspecies.

Compared to other anuran species commonly used in toxicity tests, *R. pipiens* are less sensitive to Cu exposure. LC50 concentrations reported in *Ecotoxicology of*

Amphibians and Reptiles were over twice as high for *R. pipiens* than *R. catesbeiana* or *R. palustris* under identical experimental conditions.

Although the LC50 for the initial 96-hour toxicity experiment was higher than LC50 results reported in *Ecotoxicology of Amphibians and Reptiles*, values generated in replicates of this study are consistent. LC50 values calculated in the second and third experiment fall within the 95% confidence intervals calculated for the first experiment indicating repeatability.

Due to differences in water quality and experimental design, it is difficult to compare these results to other findings. Differences in water hardness, alkalinity, and pH influence cupric ion activity, which in turn affects Cu toxicity. Erickson (1996) studied the effects of water chemistry on Cu toxicity to fathead minnows. Results of Erickson's study indicate Cu toxicity is negatively correlated with water pH, hardness and alkalinity.

The life stage at time of exposure also has a profound affect on toxic response. That larval amphibians are more susceptible to environmental contamination than either the embryo or adult phase is fairly well accepted by amphibian toxicologists. Lande's 1973 study indicates *R. pipiens* embryos are unaffected by copper sulfate concentrations that cause mortality in *R. pipiens* larvae. However, differences in toxic response between tadpoles of various ages have not been studied, which may explain discrepancies in LC50 values from different studies.

Initial LC50 experiments were designed to determine sublethal exposure concentrations for a 7-d exposure. A fundamental toxicological premise is that toxicity is a function of exposure time as well as concentration. LC50 values determined after

the 7-d exposure were lower than LC50 values for the 96-hour exposure, which is to be expected. Calculated LC50s for the 7-d exposure ranged from 0.072-0.078 mg/L Cu. This range is typical for a 96-hour exposure (Gottschalk, 1995) but there is no 7-d exposure information to compare to the values generated during this experiment.

Longer exposure periods were designed to reveal behavioral or morphological changes that had previously been observed prior to mortality in the 96-hour exposure without actually causing mortality. Examples of chemically induced behavioral/morphological changes seen in the past include: hyperactivity caused by DDT exposure (Cooke, 1970), changes in body size caused by Al (Jung, 1995), decreased swimming performance and kink of the tail caused by Zn and Cu exposure (Gottschalk, 1995). Swimming and escape behaviors enable tadpoles to evade predators encountered in an aquatic environment. Therefore, changes in morphology or behavior could potentially affect population structure.

Behavioral changes observed throughout the 7-day exposure involved lethargy, loss of equilibrium and apparent loss of appetite. These effects were perceptible at Cu concentrations of 0.036 mg/L, which is nearly 50% lower than the calculated LC50 for a 7-day exposure. Tadpoles lay on the bottom of the exposure chamber unwilling to move even after minor disturbances. When prodded, affected tadpoles would jerk sporadically before settling again on the bottom of the chamber. At times, tadpoles were observed to lie motionless on their side or back with no attempts to right themselves. Unaffected tadpoles swam about the chamber periodically or anchored themselves motionless against sides of the exposure chamber.

After the addition of food, unaffected tadpoles appeared to consume the alfalfa pellet particles voraciously attaching themselves to particles of the pellet. Lethargic tadpoles affected by Cu exposure didn't move after food was introduced to the exposure chamber. Little if any consumption occurred in concentrations nearing and above the LC50 for the 7-day exposure. Decreased consumption leading to smaller size is a common response to sublethal Cu exposure.

Foraging studies using Cu and *L. macrochirus* were performed by Sandheinrich (1989). After sublethal exposure to Cu, the fish in treatment groups were smaller than controls. Predation experiments indicated treatment fish were smaller due to reduced consumption. *L. macrochirus* in treatment groups pursued and captured less prey than control treatments.

Size was clearly affected by Cu exposure. After the 7-day exposure, tadpoles in concentrations above 0.071mg/L were about half the size of controls. Size is the driving force behind reproduction. Sexual maturity is not reached until adult frogs reach a certain size and after sexual maturity is reached, clutch size is determined by the size of female frogs. Exposure to contaminants that effect anuran size may influence entire populations by reducing clutch size.

Predation Conclusions Objective 1

A primary research hypothesis when designing this study was that sublethal Cu exposure would affect escape behavior, thereby increasing predation. This was theorized by Gottschalk (1995) and Lefcort (1998) after observing affects of heavy metals on tadpole behavior, but never tested. The actual results were counter-intuitive.

Tadpoles influenced by Cu exposure were predated significantly less than tadpoles among the control groups.

Copper exposure did not cause hyperactivity but rather lethargy, which is a common response to heavy metal exposure (Lefcort, 1998). Tadpoles in exposure groups higher than 0.071mg/L only moved when prodded or relocated from one exposure chamber to another after a solution change. Following relocation from the exposure chamber to the predation tank, exposed tadpoles sank to the bottom of the tank and remained motionless. Control tadpoles swam around the tank continuously after relocation. Once the fish were released from the screened enclosure, they waited motionless until a tadpole moved. As soon as tadpole motion was detected, the fish swam quickly and consumed the tadpole.

When designing an experiment where the results are contingent on another organism/predator, it is imperative to consider natural behaviors of that organism. Most aquatic predators are visually oriented and only strike moving prey. O'Brien's 1976 foraging study indicates *L. macrochirus* require some motion, even sinking to recognize an item as prey. Another example is the 1994 predation study performed by David Skelly using anesthetized tadpoles and dragonfly larvae. Tadpoles in motion were attacked over four times the rate of anesthetized tadpoles.

Growth Recovery Objective 2

After the 7-d toxicity experiment, tadpoles exposed to different concentrations of Cu were clearly smaller than control tadpoles. Tadpoles not used in the predation

portion of the study were reared to ascertain the length of time needed to recover with respect to body length.

Organisms have several adaptive strategies to combat stressors encountered throughout an entire life cycle. Without these strategies mass extinctions would be much more prevalent. Although recovery data on amphibians is limited, stress recovery is a well-studied topic regarding fish.

Environmental contaminants cause observable changes such as size and also biomechanical changes that are only apparent with the help of specialized analysis equipment. Reddy's 1998 study found lead exposure to change blood enzymes that affect metabolism in a common carp native to India (*Labeo rohita)*. When the fish remained in treatment concentrations, neuro-muscular changes were apparent and eventually death. Once fish were removed from lead treatment waters, perceived neuro-muscular changes reverted back to normal. Recovery with respect to the hematological parameters measured required 60 days.

Nineteen days after the tadpoles were removed from Cu treatments, there was no difference in body length between exposed and control tadpoles. However, length was the only parameter measured. Blood and tissue samples were not analyzed for changes in enzyme or protein levels that may result from Cu exposure.

Tadpoles surviving and recovering from the 7-d toxicity experiment may be less genetically diverse than the original population exposed. Recovering tadpoles may have similar genetic properties that enabled them to recover rather than perish. Schlueter (1995) found that fathead minnows (*Pimephales promelas*) surviving Cu exposure had resistant alleles that better enabled the fish to withstand Cu toxicity. This 1995 study

also suggests populations previously exposed to contaminants may be less genetically diverse than those never subjected to environmental pollutants.

Genetic diversity is considered one of the most important indications of population health. Lack of genetic diversity throughout a species places the entire population in jeopardy from disease and contaminant toxicity. *Aeromonal hydrophila* is a bacterium that has been credited with anuran population declines in Colorado (Blaustine, 1995). However, it is unclear why whole populations are suddenly being affected by this bacterium. A plausible explanation would be the loss of genetic diversity due to stressors in early embryo or larval stages.

The appearance from this study is that exposure to Cu does not have lasting effects because *R. pipiens* are able to recover with time once the stressor is removed. Copper may even be protective, inducing lethargy, which minimizes predation. However, there may be reason for concern. Genetic diversity is likely to decrease due to the stress of Cu exposure in early tadpole stages. Tadpoles surviving the stress of exposure are likely to contain similar genetic characteristics that allow detoxification of the contaminant. With a loss of genetic diversity populations may be at risk for effects of secondary stressors.

To better understand the effects metals may have on anuran populations, research should be conducted on tadpoles surviving sublethal exposure to contaminants. Detailed genetic studies may elucidate defense mechanisms that enable one individual to recover while another cannot. Contact with a bacterium or fungi after sublethal contamination exposures may be instrumental in determining effects of secondary stressors on anuran populations.

APPENDIX

RESULTS OF STATISTICAL ANALYSIS FOR THE PREDATION AND GROWTH RECOVERY EXPERIMENTS

Predation Experiment analyzed by ANOVA

Experiment	Degrees of Freedom Total	Degrees of Freedom model	F value	P value
1	23	5	2.84	0.0463
2	19	5	3.09	0.043
3	35	5	7.17	0.0002

Growth Recovery experiment analyzed by Kruskal Wallis

Group	Age days	Degrees of Freedomf	Chi Square Value	P value
1	11	2	22.91	0.0001
	18	2	16.05	0.0003
	25	2	6.28	0.005
	32	2	0.14	0.87
2	11	3	38.4	0.0001
	19	3	5.33	0.01
	25	3	7.71	0.05
	33	3	3.7	0.2
3	12	3	37.95	0.0001
	20	3	5.34	0.01
	26	3	7.68	0.05
	35	3	5.94	0.11

REFERENCES

Acker PM, Kruse KC, Krehbiel EB. 1986. Aging *Bufo americanus* by skeletochronology. *Journal of Herpetology*. 20:570-574

Agency for Toxic Substances and Disease Registry. 1990. Toxicological Profile for Copper. TP 90-08. Final Technical Report. U.S. Department of Health and Human Services. Atlanta, GA.

Axelsson E, Nystrom P, Sidenmark J, Bronmark C. 1997. Crayfish predation on amphibian eggs and larvae, *Amphibia-Reptilia* 18L217-228.

ASTM (1994) *Annual book of ASTM standards* 11.02. Philadelphia, PA, American Society for Testing and Materials.

Baxter, G. T. 1952. Notes on growth and the reproductive cycle of the leopard frog, *Rana pipiens* Schreber, in Southern Wyoming. *J. Colo. Wyoming Acad. Sci.* 4:91

Blaustein A, David B, Wake. 1995. The puzzle of declining amphibians. *Scientific American* 272(4):52-57

Bleckmann H, Lotz T, 1987. The vertebrate-catching behavior of the fishing spider *Dolomedes triton* (Araneae, Pisauridae). *Animal Behavior*. 35:641-651.

Bowen H. 1985. *The Handbook of Environmental Chemistry* Vol 1 part D: The natural environmental and the biogeochemical cycles, Springer-Verlan, New York, pp 1-26

Branch LC, Taylor DJ. 1977. Physiological and behavioral responses of larval spotted salamanders (*Amvystoma maculatum*) to various concentrations of oxygen. *Comparative Biochemistry and Physiology* 58A:269-274

Bryan M.D. Morris J.E. Atchison G.J. 1994. Methods for culturing bluegill in the laboratory. *The Progressive Fish Culturist*. 56:217-21

Burggren WW, Just JJ. 1992. Developmental changes in physiological systems, IN: Feder ME, Burggren WW, editors, *Environmental physiology of the amphibians.* Chicago IL: University of Chicago. P.467-530

Butler M.J. 1988. In situ observations of bluegill (*Lepomis macrochirus* Raf) foraging behavior: the effects of habitat complexity, group size, and predators. *Copeia*. 4:939-944

Caldwell JP, Thorp JH, Jervey TO. 1980. Predatory prey relationships among larval dragonflies, salamanders, and frogs. *Oecologia* 46:285-289

Calevro F, Campani S, Ragghianti M, Bucci S, Mancino, G. 1998. Tests of toxicity and teratogenicity in biphasic vertebrates treated with heavy metals. *Chemosphere* 37(14): 3011-3017.

Cheng, T. C. 1979. *Copper in the Environment*. Part 2 Health Effects, John Wiley and sons, Toronto, PP 401-432

Clark D, Cantu R, Cowman D, Maxson D. 1998. Uptake of arsenic and metals by tadpoles at an historically contaminated Texas Site. *Ecotoxicology* 7: 61-67

Crowder L.B. Cooper W.E. 1982. Habitat structural complexity and the interaction between bluegills and their prey. *Ecology* 63(6) 1802-1813

Daszak P. 1998. A new fungal disease associated with amphibian population declines: Recent research put into perspective. *British Herpetological Society Bulletin* 65: 38-41

Doody Sean, J. E. Young. 1995. Temporal Variation in Reproduction and Clutch Mortality of Leopard Frogs (*Rana utricularia*) in South Mississippi. *Journal of Herpetology*. 29 (4): 614-616

Duellman WE, Trueb L. 1994. *Biology of Amphibians.* Baltimore and London. The Johns Hopkins University Press. 670p.

Fichter E., A. D. Linder. 1964. *The Amphibians of Idaho,* Special Publication, Idaho State University

Fite K, Blasustein A, Bengston L, Hewitt H. 1998. Evidence of retinal light damage in *Rana cascadae:* A declining amphibian species. *Copeia* 4: 906-914

Flemming CA, Trevors JT. 1998. Copper toxicity and chemistry in the environment. *Water, Air, and Soil Pollution* 44: 143-158.

Force, E. R, 1933. The age of attainment of sexual maturity of the leopard frog, *Rana pipiens*, in northern Michigan. *Copeia* 1933:128-131

Forstner U. and G. T. Wittmann 1979. *Metal pollution in the aquatic environment*, Springer-Verlan, Berlin 386 p

Gadow H. 1908. *Through southern Mexico. Being an account of the travels of a naturalist.* London:Witherby. 527p.

Gilbert Mireille, Raymond Leclair, Rejean Fortin. 1994. Reproduction of the Northern Leopard Frog (*Rana pipiens*) in Floodplain Habitat in the Richelieu River, P. Quebec, Canada. *Journal of Herpetology.* 28(4): 465-470.

Gilhen, J. 1984. *Amphibians and Reptiles of Nova Scotia*, Nova Scotia Museum, Halifax, Nova Scotia.

Gosner K. 1960. A simplified table for staging anuran embryos and larvae with notes on identification. *Herpetologica* 16:183-190

Gottschalk J. 1995. Copper and zinc toxicity to the gray tree frog *(Hyla chroysocelis)* and the northern leopard frog (*Rana pipiens)*. MS Thesis, Clemson University, Clemson, South Carolina. 68pp.

Gundernatsch JF. 1912. Feeding experiments on tadpoles, I. The influence of specific organs given as food on growth and differentiation. A contribution to the knowledge of organs with internal secretion. *Wilhelm Roux' Arch Entwickl Orgungmech* 35:457-483

Halliday T. 1998. A declining amphibian conundrum. *Ecology* 394:418-419

Harris ML, Chora L, Bishop CA, Bogart JP. 2000. Species and age related differences in susceptibility to pesticide exposure for two amphibians, *Rana pipiens* and *Bufo americanus*. *Bulletin of Environmental contamination and toxicology* 64:263-270

Henry PFP. 2000. Aspects of amphibian anatomy and physiology In: Sparling DW, Linder G, Bishop CA. editors. *Ecotoxocology of Amphibians and Reptiles* Pensacola FL: Society of Environmental Toxicology and Chemistry (SETAC). 904p.

Herkovits Jorge, Luisa Alejandra Helguero. 1997. Copper toxicity and Copper-Zinc interactions in amphibian embryos. *The science of the total environment*. 221:1-10

Holomuzki JR. 1995. Oviposition sites and fish deterrent mechanisms of two stream anurans. *Copeia* 1995:607-613

Johnson PTJ, Lunde KB, Ritchie EG, Launer AE. 1999. The effect of trematode infection on amphibian limb development and survivirship. *Science* 284:802-805.

Jorgensen CB. 1992. Growth and reproduction. In: Feder ME. Burggren WW, editors, *Environmental physiology of the amphibians.* Chicago IL: University of Chicago. P 439-446.

Jung RE, Jagoe CH. 1995 Effects of low pH and aluminum on body size, swimming performance and susceptibility to predation of green tree frog (*Hyla cinerea*) tadpoles, *Canadian Journal of Zoology*. 73:2171-2183.

Kao L.T. Wetterer J.K. Hairston N.G. 1985. Fish size, visual resolution and prey selectivity. *Ecology* 66(6):1729-1735.

Kehr AI, Schnack JA, 1991. Predator-prey relationship between giant water bugs (*Belostoma oxyurum*) and larval anurans (*Bufo arenarum*). *Alytes* 9:61-69.

Knobeloch L, M Ziarnik, J. Howard. 1994. Gastrointestinal upsets associated with ingestion of copper-contaminated waters. *Environmental Health Perspectives*. 102: 958-961

Lande, S. Prichard, Guttman. 1973 The effects of copper sulfate on the growth and mortality rate of *Rana pipiens* tadpoles. *Herpetologica* 29(1): 22-27.

Langhelle A, Lindell M, Nystrom P. 1999. Effects of ultraviolet radiation on amphibian embryonic and larval development. *Journal of Herpetology* 33(3):449-456

Lanno, R. P., Conder, J. M., and Seals, L. Critical limits for heavy metals in soils and surface waters Workshop on Effects-Based Approaches for Heavy Metals Chapter 4.1.7 pp. 49-55

Lefcort H, Meguire RA, Wilson LH, Ettinger WF. 1998. Heavy metals alter the survival, growth , metamorphosis, and antipredatory behavior of Columbia Spotted Frog (*Rana luteiventris)* tadpoles. *Archives of Environmental Contamination and Toxicology* 35:447-456

Linder G. Ecotoxicology of Metals In: Sparling DW, Linder G, Bishop CA. editors 2000. *Ecotoxocology of Amphibians and Reptiles* Pensacola FL: Society of Environmental Toxicology and Chemistry (SETAC). P 325-459

Lopez JM and Lee GF 1977. Environmental Chemistry of copper in Torch Lake, Michigan. *Water Air and Soil Pollution.* 8: 373

Lynn G, Edelman A. 1936. Crowding and Metamorphosis in the Tadpole. *Ecology.* 17(1): 104-109

Materna Ej, Rabeni Cc, Lapoint Tw. 1995. Effects Of The Synthetic Pyrethroid insecticide, asfenvalerate, on larval leopard frogs (*Rana* spp.). *Environmental Toxicology and Chemistry*. 14(4)613

McDiarmid RW, Mitchell JC. 2000. Diversity and distribution of amphibians and reptiles. In: Sparling DW, Linder G, Bishop CA. editors. *Ecotoxocology of Amphibians and Reptiles* Pensacola FL: Society of Environmental Toxicology and Chemistry (SETAC). P 15-69.

Merrell, D.J.1977. Life history of the leopard frog *Rana pipiens*, in Minnesota. *Occ. Pap.* 15

Mitelbach G.G. 1981. foraging efficiciency and body size: A study of optimal diet and habitat use by bluegills. *Ecology*. 62(5):1370-1386.

Murphy JE, Phillips CA, Beasley VR 2000. Aspects of amphibian ecology. In: Sparling DW, Linder G, Bishop CA. editors. *Ecotoxocology of Amphibians and Reptiles* Pensacola FL: Society of Environmental Toxicology and Chemistry (SETAC). P 141-178.

Nibbelink N.P. Stephen C.R 1998. Interlake variation in growth and sixe structure of bluegill (*Lepomis macrochirus*): inverse analysis of an individual based model. *Canadian Journal of Fisheries and Aquatic Science*. 55:387-396

Nriagu JO, Pacyna JM. 1988. Quantitative assessment of worldwide contamination of air, water and soils by trace metals. *Nature.* 279:409-411

O'brien J.W. Slade N.S. Binyard G.L. 1976. Apparent size as the determinant of prey selection by bluegill sunfish (*Lepomis macrochirus*). *Ecology*. 57:1304-1310.

Phillips Kathryn. 1990. Where have all the frogs and toads gone? *BioScience* 40(6):422 424

Pickering WF 1979 in Nriagu JO editor, *Copper in the Environment* Part 1 Ecological Cycling. John Wiley and Sons Inc, New York. p217-235.

Richards C.M. 1958, The inhibition of growth in crowded *Rana pipiens* tadpoles. *Physiology and Zoology*. 31:138-151

Sanders BM, Goering PL, Jenkins K. 1996 The Role of general and metal specific cellular responses in protection and repair of metal induced damage: Stress proteins and metallothioneins In: Magos L, Suzuki T editors *Toxicology of Metals* Lewis Publishers Boca Raton, FL.

Sandheinrich MB, Atchison GJ. 1989. Sublethal copper effects on bluegill *Lepomis macrochirus*, foraging behavior. *Canadian Journal of Fisheries and Aquatic Science*. 46:1977.

SAS Institure Inc, *SAS/STAT User's Guide*, version 6, Fourth edition, Volume 2, Cary, NC:SAS Institute Inc. 1989. 846 p.

Scott-F Janeck J., Paul Henning Krigh, Jason Weeks. 1997. Sublethal toxicity of copper to a soil-dwelling springtail (*Folsomia fimetria*) (Collembola: Isotomidae). *Environmental Toxicology and Chemistry.* 16(12) 2538-2542

Shumway Waldo. 1940 Stages in the Normal Development of *Rana pipiens*. *Anatomical Record.* 78 (2): 139-147

Skelly DK. 1994. Activity level and the susceptibility of anuran larvae to predation. *Animal Behavior* 47:465-468

Stein B, Flack S. 1997. The state of U.S. plants and animals. *Environment* 39(4):7-11, 34-39

Tupa DD, Davis WK. 1976 Population dynamics or the San Marcos Salamander, *Euryces nana* Bishop. *Texas Journal of Science*. 27:179-195.

Taylor Cecil, Jerry Kollros. 1945. Stages in the Normal Development of *Rana pipiens* Larvae. *Anatomical Record*. 94: 7-23

WEST, Inc, and Gulley, David D. 1994. Toxtat 3.4. Cheyenne, WY

Wells K. 1977. The social behavior of anuran amphibians. *Animal Behavior* 25:666-693

Wright Mary, Linda Blanchard, Suzanne Jorey, Carolyn Basso, Yvonne Myers Christine Paquette. 1990. Metamorphic Rate as a Function of the Light/Dark Cycle in *Rana pipiens* Larvae. *Comp. Biochem and Physiology*. 96(1): 215-220.